UNIVERSITY OF TASMANIA

Tissue specificity of cytosolic K^+ retention, Na^+ extrusion, and vacuolar Na^+ sequestration traits in the context of differential salinity stress tolerance in barley and wheat

by

Honghong Wu

School of Land and Food

M.Sc.

Nanjing Agricultural University, China

B.Sc.

Jiangxi Agricultural University, China

Submitted in fulfilment of the requirements for the Doctor of Philosophy

University of Tasmania

November 2015

Declarations

Declaration of Originality

"This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright."

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Statement Regarding Published Work Contained in Thesis

"The publishers of the papers comprising Chapters 3 to 7 and Appendix 1 and 2 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968."

Honghong Wu
University of Tasmania
November 2015

Statement of Co-authorship

This thesis was completed during my PhD study in the School of Land and Food at the University of Tasmania. This thesis contains no experimental results that have previously presented for any degree at this or other institution.
The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Honghong Wu, University of Tasmania
Sergey Shabala, University of Tasmania
Lana Shabala, University of Tasmania
Meixue Zhou, University of Tasmania
Min Zhu, University of Tasmania
Jayakumar Bose, University of Tasmania
Karen Barry, University of Tasmania
Stefano Mancuso, University of Florence
Elisa Azzarello, University of Florence
Camilla Pandolfi, University of Florence
Zhonghua Chen, University of Western Sydney
Xiaohui Liu, University of Western Sydney
Giovanni Stefano, Michigan State University

Author details and their roles:

Paper 1: **Wu H**, Shabala L, Barry K, Zhou M, Shabala S (2013) Ability of leaf mesophyll to retain potassium correlates with salinity tolerance in wheat and barley. *Physiologia Plantarum* **149**: 515–527. --- Located in *Chapter 3*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. S.S., H.W., and L.S. wrote the manuscript. H.W., S.S., and K.B. conducted experiments. H.W. and S.S. analysed data. L.S. and M.Z. contributed to results interpretation and provided critical analysis of the manuscript.

Paper 2: **Wu H**, Zhu M, Shabala L, Zhou M, Shabala S (2015) K^+ retention in leaf

mesophyll, an overlooked component of salinity tolerance mechanism: a case for barley. *Journal of Integrative Plant Biology* **57**: 171–185. --- Located in *Chapter 4*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. S.S. and H.W. wrote the manuscript. H.W., and Z.M. conducted experiments. H.W. and S.S. analysed data. L.S. and M.Z. contributed to results interpretation and provided critical analysis of the manuscript.

Paper 3: **Wu H**, Shabala L, Zhou M, Shabala S (2014) Durum and bread wheat differ in their ability to retain potassium in leaf mesophyll: implications for salinity stress tolerance. *Plant and Cell Physiology* **55**: 1749–1762. --- Located in *Chapter 5*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. H.W. and S.S. wrote the manuscript. H.W. conducted experiments. H.W. and S.S. analysed data. L.S. and M.Z. contributed to results interpretation and provided critical analysis of the manuscript.

Paper 4: **Wu H**, Shabala L, Zhou M, Stefano G, Pandolfi, C, Mancuso S, Shabala S (2015) Developing and validating a high-throughput assay for salinity tissue tolerance in wheat and barley. *Planta* **242**: 847–857. --- Located in *Chapter 6*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. S.S. and H.W. wrote the manuscript. H.W., G.S., and C.P. conducted experiments. H.W. analysed data. L.S., M.Z., and S.M. contributed to interpretation of results and provided a critical analysis of the manuscript.

Paper 5: **Wu H**, Shabala L, Liu X, Azzarello E, Pandolfi, C, Zhou M, Chen, ZH, Bose J, Mancuso S, Shabala S (2015) Linking salinity tolerance with tissue-specific Na^+ sequestration in wheat roots. *Frontiers in Plant Science* **6**: 71. --- Located in *Chapter 7*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. H.W. and S.S. wrote the manuscript. L.S., H.W., X.L., E.A., and C.P. conducted experiments. H.W. and S.S. analysed data. L.S., M.Z., C.Z., J.B., and S.M. contributed to results interpretation and provided critical analysis of the manuscript.

Paper 6: **Wu H,** Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S: Cell-specific regulation of ionic homeostasis in the context of salinity stress tolerance: a case study for root elongation zone. *Plant Physiology* (Submitted) --- Located in *Chapter 8*

Author contribution: H.W. and S.S. conceived and designed the research. H.W. and S.S. wrote the manuscript. H.W. and L.S. conducted experiments. H.W. and S.S. analysed data. L.S., M.Z., E.A., and S.M. contributed to results interpretation and provided critical analysis of the manuscript.

Paper 7: **Wu H**, Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S: Comparative analysis of Na^+ exclusion and sequestration patterns in roots of durum and bread wheat in the context of the long-distance salt stress signaling and adaptation. *New Phytologist* (Submitted). --- Located in *Chapter 9*

Author contribution: H.W. and S.S. conceived and designed the research. H.W. and S.S. wrote the manuscript. H.W. and L.S. conducted experiments. H.W. and S.S. analysed data. L.S., M.Z., E.A., and S.M. contributed to results interpretation and provided critical analysis of the manuscript.

Paper 8: **Wu H**, Shabala L, Zhou M, Shabala S (2015) MIFE technique-based screening for K^+ retention in the leaf mesophyll as a tool for crop breeding for salinity stress tolerance. *Bio-Protocol* **5**: 1–10. --- Located in *Appendix 1*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. H.W. and S.S. wrote the manuscript. H.W. conducted experiments. H.W. and S.S. analysed data. L.S. and M.Z. provided critical analysis of the manuscript.

Paper 9: **Wu H**, Shabala L, Zhou M, Shabala S (2015) Chloroplast-generated ROS dominates NaCl-induced K^+ efflux in wheat leaf mesophyll. *Plant Signal Behav* **10**: 5, e1013793. --- Located in *Appendix 2*

Author contribution: S.S., L.S., M.Z., and H.W. conceived and designed the research. H.W. and S.S. wrote the manuscript. H.W. conducted experiments. H.W. and S.S.

analysed data. L.S. and M.Z. contributed to results interpretation and provided critical analysis of the manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Student signature	Primary supervisor signature
Honghong Wu	Prof Sergey Shabala

Acknowledgements

In 1987, I was born in a town named “Youdunjie” in Jiangxi province in middle China. Since then, I grew up and finished my high school education in hometown in “Youdunjie middle school” until June 2004. At this year, I passed the “National Higher Education Entrance Examination” and was enrolled in Jiangxi Agricultural University to study in the major “Biotechnology (Microbiology)”. I definitely miss the 4 years Uni-life of undergraduates. At September 2008, I became a master in research student (majored in Biochemistry and Molecular Biology) in Nanjing Agricultural University. In Nanjing, I met with Prof Wenbiao Shen, and became a master student under his supervision. At this stage I received a good training and learned the principals in how to conduct scientific research.

In September 2011, I moved to University of Tasmania (UTAS) to start as a research associate in Prof Sergey Shabala’s laboratory after completing my master study. One year later (November 2012), I was enrolled in School of Agricultural Science at UTAS to study in PhD in Agricultural Science. I would like to express my special thanks to my fantastic supervisory team: Prof Sergey Shabala, A/Prof Meixue Zhou, and Dr Lana Shabala. Thanks for their fantastic supervision, instruction, and support in my PhD study. Their selfless input is really a big help in my PhD study. Without the selfless help from them, I couldn’t have all the achievements I have gained in PhD study. During my PhD study, I can access to them at any working time and also the spare time even the weekend. I still remember Sergey and Lana even dealing with my emails at airport. Meixue send me the feedback of manuscript at the midnight of Saturday. With such excellent supervisors, I would say it is my luck to be supervised by them. I really appreciate it.

I would like to thank School of Agricultural Science for offering me the PhD scholarship. Without this important financial support, I might be not able to start my PhD study at University of Tasmania.

I would like to thank the GRDC (Grain Research and Development Corporation of Australia) for the funding support to my project.

I would like to thank all the colleagues in our laboratory, e.g. postdoc Edgar Bonales-Alatorre, Jayakumar Bose and Maheswari Jayakannan, Dr. Min Zhu, Mr. Xin Huang, Mr. Joseph Hartley, Mrs. Norhawa Puniran Hartley, Mr. Shivam Sidana, Dr. William Percey, Ms. Zhihous Falakboland, Ms. Haiyang Wang, Ms. Feifei Wang, Ms. Yanling Ma, Mr.

Billal Gill, Dr. Mohammad Al Nayef, Dr. Getnet Adem, and Dr. Suresh Panta etc. Thank you for the helps and supports during my PhD study. I also miss the KFC and Pizza night, and the Pizza and Movie night.

I would like to thank my friends in Hobart, e.g. Dr. Shi Feng, Ms. Peipei Zhang, Mrs. Fang Xia, Dr. Wei Hu, Mr. Wei Hong, Dr. Yaping Liu, Mr. Huaming An, and Mr. Alvin Lam etc. I miss the time we spent together for entertainments, BBQ, and Hotpot dinner etc.

I would like to thank some staffs in School of Agricultural Science, e.g. Mrs. Sally Jones, Mr. Robert Brockman, Mr. Andrew Measham, Mrs. Angela Richardson, Mr. Philip Andrews etc. A special thanks for Dr Peter Johnson for his help of Englsih usage check of this thesis. Thank you all for the helps and supports during my PhD study.

I must express my special thanks to my family (my mum, dad, sister, and brother). Without their understanding, support, patience and encouragement, I could not able to finish my PhD study in three years. I thank you all for your love and endless support.

Last but not least, I will miss the important journey (my PhD study) in my life. Also, I will miss lovely you all (my supervisors, friends, and colleagues) I have met in Australia. Once again, thank you all for the help and support in my PhD study and life.

It is the time to say “see you” to all of you, but I always believe one day we definitely will meet again.

Honghong Wu

20-11-2015, Hobart

致谢

---写在博士毕业之际的话

光阴荏苒，岁月如梭！如今年近而立之年的我却是真真切切的理会到了这个儿时只会觉得有些老生重弹的话语。从年少不识愁滋味的悠闲，到如今历久弥坚、一路向前的踏实，回首看去，这些年来的求学路上有过懵懂，有过挫折，却也有过喜悦，有过收获；有过迷茫，有过低落，也更曾有过坚定，有过激情。这些年一路走来，有太多太多的话想说，却也有太多太多的感情无法去一一细表而只能是自己孤独地品尝。

1987 年我出生在中国江西省鄱阳湖边的一个小镇油墩街镇上。小时候在幼儿园调皮捣蛋的经历现在依稀还能记起一些。五岁的时候我进入小学学习，十岁进入初中学习，十三岁进入高中学习，直到十六岁第一次参加完高考。这期间十一年的学习对我而言更多是是一种处于懵懵懂懂的状态中的被动进行的学习，除了学校规定的学习任务外，印象中并没有做过什么太多的课后作业，课外补习等等，反倒是金庸、古龙以及梁羽生等大家名家的武侠小说以及琼瑶、席慕蓉等的言情小说基本看了个遍。就这样，第一次懵懵懂懂的参加完高考后，考试成绩较差，只达到了当年的专科院校的录取线，自己都感觉到很是不好意思。加上后续陆续发生的其它的一些事情也让自己比较强烈的意识到高考是一个分水岭，之前大家都可以嘻嘻哈哈无忧无虑在自己的小天地里信马由缰，然而高考完之后要面对却是一个向我们敞开怀抱的社会。高考完之后，同学之间的路已经不一样了。所以我义无反顾的复读了一年，所幸皇天不负有心人，经过一年的紧张主动的积极学习，第二次高考我的成绩相对已经比较可以接受，已经超出当年二本录取线一大截。也由此，在我姐姐的影响下（姐姐当时正在江西农业大学学习），我没有多做犹豫二本第一志愿填报了江西农业大学的生物技术专业（因为高二开始已经对生物这门课程比较感兴趣）。也由此，我人生中的在老家度过的阶段画上了一个句号（很高兴我这段稚嫩的童年及青春能够在老家留存，也因此我的脑海中能一直有魂牵梦绕的老家印象），异地求学的阶段也即将正式开启。“长风破浪会有时，直挂云帆济沧海”，我一直笃定并坚韧的向前路进发。

从 2004 年九月进入大学学习以来，11 年美好青春的光阴已经洒在了我人生经历的时间长河里。我的脑海中，回忆也如流水般一波波的汹涌着浪花。17 岁收拾行囊，离开爸妈以及大家庭独自到大学报道求学，那种雀跃难耐的心情至今想起还会在心里泛起涟漪。那时，离乡的行囊里装着的是自己的无知无畏和年少不识愁。大学本科四年的光阴是那么美好而心无他念，至今想起还会在心里感恩本科阶段的母校–江西农业大学。感谢我的江农老师们（例如本科毕业设计指导老师-理学院王义华教授、考研推荐老师-农学院朱昌兰教授、本科班主任-生工院魏赛金教授等）教会我的除了专业知识外的其他做人做事的道理以及一脉相承的传承下来的“抱朴守真”的江农精神。我至今还记得当年一系列的新生座谈会上生工院书记吴文军教授说的一句话：“没有思想上的清醒就没有行动上的坚定”，以及当年的生工院院长涂国全教授以他自身留学英国以及投身科研的经历来激励大家求知求学的映像。感谢室友们（23 栋 603 室的张志福、杨键、张平）、同班同学们（李志会、陈刚、徐学琪、黎前明、王毅飞、蔡林、钟建春等）四年时间的朝夕相处。另外，对于所有在江西农大的四年时光里直接或间接帮助过我的老师（院办彭剑锋老师等）、同学（生工班的张涛同学等）或是好心人，谨于此处一并致谢。谢谢你们四年的陪伴！有了你们我的本科四年的生活才会那么回味悠远。

历经四年求学江农之后，2008 年 9 月，我如愿的进入了研究生阶段进行下一阶段的学习深造。这一次，我来到了古城金陵的南京农业大学，师从生科院沈文飚教授。三年的时光也是如风一般飞逝而去，其中的酸甜苦辣、拼搏的汗水、失败的苦闷、收获时的喜悦眼泪等等不一而足，难以一一名状。师门严格的要求、高标准的培养让我受益至今。“诚朴勤仁”的南农精神也让我受益匪浅。宝剑锋从磨砺出，梅花香自苦寒来。而没有真正经历其中苦味的人却是无法道出其中的韵味和香味的。感谢沈文飚老师的身先示范的教导和鞭策鼓励及肯定支持，从沈老师身上我体会到了并一直在学习着他的拼命三郎式的工作激情、踏踏实实的科研态度、敢于质疑的科研精神。感谢师兄师姐们（凌腾芳师兄，徐晟师兄、崔为体师兄、曹泽彧师兄、吴婷婷师姐、谢彦杰师兄、黄晶晶师姐、韩毅师兄、宣伟师兄、孙永刚师兄等），同门们（韩斌、付广青、李梅月），师弟师妹们（周兴虎师弟、肖笑师妹、方涛师弟、林玉婷师妹），以及黄本开（开哥），卢晗的帮助和鼓励。我永远记得 2008 年 3 月刚去南农熟悉实验室时，那时经常晚上跟着实验室的凌腾芳师兄、徐晟师兄、黄晶晶师姐、开哥等人一起去校门外的夜宵摊吃烧烤、喝啤酒的场景。当

然，在南农的三年时光里，我也需要感谢南农的其他的一些老师们（生科院徐朗莱教授、院办康若依老师、食品院黄明教授等）和同学朋友们（如舍友韩斌、高可辉、王卫青，同学林健、张强、洪永波、陈禹等）以及工作生活上碰到的一些好心人的支持、鼓励和帮助，谨于此处一并致谢。谢谢你们三年多的陪伴！

时间转眼来到了 2011 年，这一年我 24 岁了。对我而言，这是我人生中的另外一个重要时间节点。这一年九月，我怀揣着理想和梦想，飘扬过海来到了万里之外的异国他乡–澳大利亚，来到了塔斯玛尼亚大学求学。这一年，离乡的行囊里除了对未来的憧憬外，却也第一次打上了一丝思乡的烙印。蓝天白云、异国他乡，当我踏足这片土地的时候我的心情是激动复杂而难以言说的。四年多以来，我先是作为 research associate 在 Sergey Shabala 教授实验室工作了 14 个月，直到 2012 年 11 月正式注册塔大农学院农业科学博士生课程。这四年多的时间里，我非常珍惜这来之不易的学习机会，尽自己所能，把主要精力都集中在了学习上。回首这四年多的塔大时光，我可以自豪的说，我做到了无愧于心。于学校而言，我超量两三倍的完成了培养任务里的规定，给出了一份无可置喙的答卷；于导师们而言，我超质超量的完成了课题任务，达到了培养目标；于家人亲友而言，我成功的完成了博士学业；于我个人而言，我成功的升华了自我，却也发现与未知的生物世界相比自己还只是其中的探索者中非常渺小的一员。

塔大求学生涯的这四年的时光里，拼搏、再拼搏，奋斗、再奋斗，迷茫过，激情过，失落过，喜悦过，失败过，收获着，所有的所有现在回首总结却发现都更多的是对自身的一种锤炼，一种升华。“试玉要烧三日满，辨材须待七年期”，我只想说这四年多我没有辜负我的大好青春年华，这四年多我也没有后悔过我的选择，这四年多我也一直在感悟学习进步着。所有的所有，我都要感谢很多人。首先要感谢的是我的导师 Sergey Shabala 教授、周美学副教授及 Lana Shabala 博士和塔大农学院提供的来之不易的全额奖学金学习机会。非常感谢在这四年的时间里他们对我的学习上生活上的周到的安排和细心的关怀。我所有成绩的取得都离不开他/她们的谆谆教导、积极鼓励和大力支持。他/她们渊博的学识，严谨的工作态度、对待科学的热情和好奇心，以及永无止境的拼搏精神都为我树立了好榜样。我还记得总是能第一时间得到导师 Sergey Shabala 教授的不分工作日与否的回复以及在讨论科学问题时对我的启发和循循诱导，也还记得有时周末晚上甚至凌晨 1 点多还能收到周美学老师的对我的稿件的详细修改，也还能记得 Lana Shabala 博士有几次在机场

还在发邮件回复解答我的一些学术问题。“见贤而思齐”，我的这些非常优秀的导师们为我在未来的工作生活中的努力方向树立了一个好标杆。

其次我要感谢的是实验室的同事们，博后 Edgar, 博后 Jay, 博后 Mahe，朱敏博士，黄鑫、Joe、Hawa、Shivam、Will 博士、Zhinous、王海洋、王菲菲、马艳玲、Billal、Mohammad 博士、Getnet 博士、Suresh 博士等。谢谢他们的帮助和相互鼓励支持，有了他们我的四年海外求学时间并不孤独。在此我要特别感谢的是我的一些朋友们，塔斯玛尼亚大学澳洲食品安全中心的冯适博士、张培培博士、人文院的夏芳、商学院的忽炜博士、地质院的洪为以及访学的刘亚平老师、工程院的安华明、植物科学学院的 Alvin 等等等等。有了你们的友情，异国他乡求学的四年时间里我也能经常感受到属于友谊的温暖力量。我总能记起大家一起包饺子、吃火锅、出去踏青的情景，也还记得我们在实验室做实验做到较晚的时候三两人之间的 pizza and KFC night, 以及经常在 Joe 和 Hawa 家举行的实验室成员间的 movie night。最难以忘怀的就是和小伙伴们在我住的地方或是芳姐家一起聚餐或做饭或吃火锅时的场景，以及和冯适、黄鑫、张培培、陈婧等人在“三号聚义厅”（学校的 learning hub 里的一个单独的学习室，因为我们经常在那里举行活动，所以私下里把它称为“聚义厅”）的欢乐时光，那时大家经常一起天南地北、天马行空的交流、一起放松下来的玩猜字等各种游戏！谢谢你们！谢谢大家四年多来的陪伴！同时我还要感谢的是在这异国求学四年多时光里曾经直接或间接帮助过我的一些老师（院办老师 Sally Jones, Robert Brockman，Angela Richardson，院实验室总管理人 Andrew Measham，院园艺中心老师 Philip Andrews 等）以及其他的好心人们。尤其是塔大农学院的 Dr Peter Johnson 对本论文英语文法上的修正。谢谢你们！有了你们的无私帮助，异国求学的四年多时间里我才能更好地做出并取得现有的成绩。一切的一切，我都怀着一颗感恩的心。在今后的路上，我会更加努力地去提高自己，争取做出更好更多的成绩。我想这应该也是对你们的友善关注和无私帮助的回报之一。同时，这里也非常感谢澳大利亚 GRDC（Grain Research and Development Corporation）基金对我的研究课题的资助。

再次，我还要感谢的是在我求学和科研路上给予我帮助的老师及朋友们。衷心感谢兰州大学南志标院士对晚辈我的提携指导，也谢谢兰州大学草地农业生态系统国家重点实验室的王锁民教授、张金林教授等老师们对我的鼓励支持。此外，我还需要感谢的是安徽农业大学茶树生物学与资源利用国家重点实验室的 Dr.张显晨老

师，三篇合作 SCI 论文的发表已经见证了我们间的科研协作所结出的累累硕果。谢谢你们大家对我的支持。其他在我求学和科研路上帮助过我的人，谨于此处一并致谢！科研奋斗的路上和你们在一起，风景必然更加精彩和不同。

最后我必须要感谢的是我的家人：爸爸、妈妈、姐姐及哥哥。有了他们的理解、鼓励和支持，我的求学之路才可以心无旁骛、一心向前。爸妈，这些年你们辛苦了。姐姐、哥哥，谢谢你们的支持。有了你们的坚定大后方以及不断的鼓励和支持，我的求学之路才能这么一路的走下来。谢谢你们！对于你们的无私的理解和全力的支持，我想我已经用实际行动证明了自己。我现在所取得的成绩是我可以拿出来的最实在的汇报，也是对你们这些年来的鼎力支持的最好的回报。爸、妈，你们的儿子，姐姐、哥哥，你们的弟弟可以让你们自豪地大声说：这些年来，他追寻着自己的心做到了自己的承诺！他没有辜负自己的大好青春年华！

写在最后的话：大学阶段以来的 11 年多的美好青春，我都把它奉献给了我自己为之欢喜而着迷的探索未知的生命科学世界的旅程而因此很少或是无暇去顾及感情或是其它。回首往事，涓滴在心，然天涯路远，知己难觅，难觅知己。然而，掬一捧清水，道三千我思，我却还愿意继续在探索未知的生命科学领域这条漫漫长路上继续地去追逐自己的理想和兴趣、却也同时会去寻觅那个冥冥中应是早已缘定的前路知己。路漫漫其修远兮，而我愿意继续去努力求索。

吴洪洪

2015 年 11 月 20 日

于 Hobart, Australia

Publications arising from this Thesis

Journal Publications

1. **Wu H**, Shabala L, Zhou M, Stefano G, Pandolfi, C, Mancuso S, Shabala S (2015) Developing and validating a high-throughput assay for salinity tissue tolerance in wheat and barley. *Planta* **242**: 847–857
2. **Wu H**, Shabala L, Liu X, Azzarello E, Pandolfi, C, Zhou M, Chen, ZH, Bose J, Mancuso S, Shabala S (2015) Linking salinity tolerance with tissue-specific Na^+ sequestration in wheat roots. *Frontiers in Plant Science* **6**: 71
3. **Wu H**, Zhu M, Shabala L, Zhou M, Shabala S (2015) K^+ retention in leaf mesophyll, an overlooked component of salinity tolerance mechanism: a case for barley. *Journal of Integrative Plant Biology* **57**: 171–185
4. **Wu H**, Shabala L, Zhou M, Shabala S (2015) Chloroplast-generated ROS dominates NaCl-induced K^+ efflux in wheat leaf mesophyll. *Plant Signal Behav* **10**: 5, e1013793
5. **Wu H**, Shabala L, Zhou M, Shabala S (2015) MIFE technique-based screening for K^+ retention in the leaf mesophyll as a tool for crop breeding for salinity stress tolerance. *Bio-Protocol* **5**: 1–10
6. **Wu H**, Shabala L, Zhou M, Shabala S (2014) Durum and bread wheat differ in their ability to retain potassium in leaf mesophyll: implications for salinity stress tolerance. *Plant and Cell Physiology* **55**: 1749–1762
7. **Wu H**, Shabala L, Barry K, Zhou M, Shabala S (2013) Ability of leaf mesophyll to retain potassium correlates with salinity tolerance in wheat and barley. *Physiologia Plantarum* **149**: 515–527
8. Shabala S, **Wu H**, Bose J (2015) Salt stress sensing and early signalling events in plant roots: current knowledge and hypothesis. *Plant Science* **241**: 109–119

Submitted

9. **Wu H,** Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S: Cell-specific regulation of ionic homeostasis in the context of salinity stress tolerance: a case study for root elongation zone. *Plant Physiology* (Submitted)
10. **Wu H**, Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S: Comparative analysis of Na^+ exclusion and sequestration patterns in roots of durum and bread

wheat in the context of the long-distance salt stress signaling and adaptation. *New Phytologist* (Submitted)

Conference Papers

1. **Wu H,** Shabala L, Bose J, Zhou M, Shabala S (2015) Sensing and signaling salt stress in plant roots. 2nd international conference on "Physiological, Biochemical, and Molecular Arguments for Salt Tolerance", 11 – 14 October, 2015, Doha, Qatar
2. **Wu H,** Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S (2015) Vacuolar Na^+ sequestration but not Na^+ exclusion from uptake confers salt tolerance in barley. SEB Prague conference, 30 June–03 July 2015, Prague, Czech Republic
3. **Wu H**, Zhu M, Shabala L, Zhou M, Shabala S (2014) Sodium and potassium nutrition in the context of the salinity stress tolerance in cereals. ComBio 2014, 28 September – 02 October 2014, Canberra, Australia
4. Zhu M, **Wu H**, Zhou M, Shabala L, Shabala S (2014) Linking osmotic adjustment and stomatal characteristics with salinity stress tolerance in barley. ComBio 2014, 28 September – 02 October 2014, Canberra, Australia
5. Shabala S, Bose J, Shabala L, Zeng F, **Wu H**, Zhu M, Adem G, Panta S, Percey W, Zhou M. (2014) Abiotic stress tolerance and crop yield: a physiologist's perspective. ComBio 2014, 28 September – 02 October 2014, Canberra, Australia
6. **Wu H**, Shabala SN, Zhou M, Shabala L. Quantifying contribution of SOS1 and GORK plasma membrane transporters towards salinity tolerance in barley by the MIFE technology, The 16th Australian Barley Technical Symposium, 08 – 11 September 2013, Melbourne, Australia
7. Shabala SN, **Wu H**, Shabala L, Zhou M. Functional genomics of salinity stress tolerance in barley assessed by the MIFE™ technology, pp. 20 – 25. VIR conference – "Modern methods of use of genetic resources in breeding barley and oats", 01 – 05 July 2013, St Petersburg, Russia

Table of Contents

List of Abbreviations

AKT/KAT, Arabidopsis inward K^+ channel

AO, non-enzymatic antioxidant systems

ANOVA, analysis of variance

ADP, adenosine diphosphate

ATP, adenosine triphosphate

ATPase, enzymes catalyse the decomposition of ATP into ADP and Pi

BSM, basic salt medium

BSTFA, N, O-bis-trimethylsilylacetamide

C4, C4 carbon fixation

CBL, calcineurin B-like proteins

CCC, cation chloride cotransporter

CCI, chlorophyll content index

cDNA, complementary deoxyribonucleic acid

CHX, cation/hydrogen exchanger

CIPK, CBL-interacting protein kinases

DA-NSCC, depolarization activated nonselective cation channel

DH, doubled haploid

DNA, deoxyribonucleic acid

DPI, diphenylene iodonium

DW, dry weight

EGCG, (−)-Epigallocatechin gallate

ER, endoplasmic reticulum

ESEM, environmental scanning electron microscope

FAO, Food and Agriculture organisation of the United Nations

FV, Na^+ permeable fast-acitivating tonoplast channels

F_v/F_m, maximal quantum efficiency of PS II

FW, fresh weight

GCMS, gas chromatography-mass spectrometry

Gd^{3+}, gadolinium chloride

GORK, guard cell outward rectifying K^+ channel

H_2O_2, hydrogen peroxide

HA, H^+-ATPase

HA-NSCC, hyperpolarization activated nonselective cation channel

HAK, high affinity K^+ transporter

HAK/KUP, high affinity K^+ transporter

HKT, high affinity K^+ transporter

KOR, K^+ outward rectifying channel

LCT, low-affinity cation transporter

LIX, liquid ion exchanger

LSCM, laser scanning confocal microscopy

MAS, maker assisted selection

MES, 2-(N-morpholino) ethanesulfonic acid

MIFE, microelectrode ion flux estimation system

MP, membrane potential

MV, methyl viologen

NAPDH, nicotinamide adenine dinucleotide phosphate

NHX1, Na^+/H^+ exchanger

NIL, near isogenic line

NSCC, nonselective cation channel

$O_2^{\cdot}$, superoxide anion

$OH^{\cdot}$, hydroxyle radicals

PCD, programmed cell death

PCR, polymerase chain reaction

PM, plasma membrane

PSII, photosystem II

PVC, polyvinyl chloride

QTL, quantitative trait locus

RNA, ribonucleic acid

ROS, reactive oxygen species

ROI, region of interest

RWC, relative water content

SEM, cryo-scanning electron microscopy

SOS1, salt overly sensitive 1, Na^+/H^+ antiporter

SOS2, salt overly sensitive 2, a serine/threonine protein kinase

SOS3, salt overly sensitive 3, a myristoylated calcium binding protein

SV, slow-activating vacuolar channels

SWC, shoot water content

TEA, tetraethylammonium chloride

TMS, tri-methyl-silyl

TPK, tonoplast K^+-permeavle channels

TUNEL, terminal deoxynucleotidyl transferase DUTP nick end labeling

Vanadate, sodium orthovanadate

V-ATPase, vacuolar type H+-ATPase

VI-NSCC, voltage independent nonselective cation channel

VP, vacuolar pyrophosphotase

WT, wild type

Abstract

Wheat and barley are ranked the second and fifth most important crops in terms of dry matter production. Both of them are classified as glycophytes, and their production is strongly affected by soil salinity. Thus, given the extent of land salinization in the world and predicted population growth to 9.3 billion by 2050, creating salt tolerant wheat and barley germplasm remains one of highest priorities for breeders.

Salinity tolerance is a complex physiological trait composed of numerous sub-traits controlled by multiple regulatory pathways. Until now, most studies were focused on traits related to sodium, such as Na^+ exclusion from uptake, control of xylem Na^+ loading, Na^+ retrieval from the shoot or vacuolar Na^+ sequestration. However, it is not Na^+ but the K^+/Na^+ ratio in the cytosol that ultimately determines plant performance under saline conditions. In recent years, K^+ retention in root mature zone has emerged as an important component of salt tolerance mechanisms in many plant species. However, whether the importance of cell's ability to maintain K^+ in plant overall salt tolerance can be extrapolated to other root zones or tissues (e.g. leaves) remained obscure prior to this work. Also elusive remained the essentiality of root Na^+ exclusion and vacuolar Na^+ sequestration in various root tissues. Furthermore, the relative contribution of each of the above salt tolerant mechanisms towards the overall salinity tolerance remained unclear, especially at the tissue specific level.

Supported by the Grain Research and Development Corporation of Australia, this work was designed to fill the above gaps in our knowledge by addressing the following specific aims:

1) to investigate the role of mesophyll K^+ retention in the overall salt tolerance in wheat and barley and to reveal the molecular and physiological identity of ion channels involved in mediating NaCl-induced K^+ efflux from leaf mesophyll cell in these species;

2) to investigate the role of leaf tissue tolerance in the overall salt tolerance in wheat and barley and its relative contribution in each species;

3) to elucidate the role of the tissue specific Na^+ distribution in the cytosol and vacuole of various root zones and its function in salt stress signalling and adaptation;

4) to quantify the relative contribution of root Na^+ exclusion from the cytosol, vacuolar Na^+ sequestration, and cytosolic K^+ retention in the overall salt tolerance at the tissue specific level;

5) to develop rapid and reliable screening methods of evaluation some key physiological traits conferring salinity stress tolerance to be used in breeding programs.

To accomplish the above goals, a broad range of electrophysiological, molecular, physiological, pharmacological, and imaging experiments was conducted to investigate the relationship between the overall salt tolerance and the above mentioned components of salt tolerance mechanisms at various levels of plant structural organisation. Our results showed that mesophyll K^+ retention is an important (and previously overlooked) component of salt tolerance mechanisms in both wheat and barley. Significantly less NaCl-induced K^+ efflux from leaf mesophyll occurred in bread wheat compared with durum wheat. Strong and positive correlation ($r = 0.64$, $P < 0.001$) was found between mesophyll K^+ retention ability and overall salt tolerance in wheat. This is consistent with the general concept that bread wheat is more salt tolerant than durum wheat. Similar results were also obtained for barley, in which a significant positive correlation ($r > 0.55$, $P < 0.01$) was found between mesophyll K^+ retention and the overall salt tolerance. Pharmacological experiments revealed, however, that NaCl-induced K^+ efflux in mesophyll was mediated by different transport systems in wheat and barley. In wheat, non-selective cation channels (NSCC) mediated NaCl-induced K^+ efflux from leaf mesophyll, whereas in barley this efflux occurred predominantly through the depolarization activated outward-rectifying K^+ channels (KOR). Chloroplast-generated reactive oxygen species (ROS) dominated the process of NaCl-induced K^+ efflux in wheat mesophyll. It is concluded that targeting mesophyll K^+ retention is an unexploited resource and might be a promising way to improve salt tolerance in both wheat and barley. A high throughput screening method was also developed based on Microelectrode Ion Flux Estimation technique (MIFE).

The use of a whole-plant phenotyping has a potential caveat of ignoring genotypic variability in the plant's ability to control Na^+ delivery to the shoot. To eliminate the possible confounding effect of roots, excised wheat and barley leaves were immersed into 50 mM NaCl solution mimicking salt accumulation in the apoplast in field plants grown under saline conditions. Significant positive correlation ($r > 0.50$, $P < 0.01$) between relative chlorophyll content index (CCI) and the overall salt tolerance was found for barley. This correlation was largely due to preferential accumulation of Na^+ in mesophyll cell vacuoles of tolerant varieties, while in sensitive varieties substantial quantities of Na^+ were detected in chloroplasts (revealed by Sodium Green fluorescence imaging). To a great surprise, strong *negative* correlation ($r = 0.77$, $P < 0.001$) between relative

chlorophyll content index (CCI) and the overall salt tolerance was found in 45 wheat varieties, even in separate clusters of bread (r = 0.48, $P < 0.05$) and durum wheat (r = 0.59, $P < 0.01$). These results indicate that the lack of effective Na^+ exclusion ability in salt sensitive wheat varieties is compensated by their better ability to handle Na^+ accumulated in the shoot via tissue-tolerance mechanisms. This work has also suggested that changes in relative chlorophyll content (measured as CCI) in excised leaves can be used as a reliable indicator of leaf tissue tolerance.

Root Na^+ exclusion was deemed as an important component in plant salt tolerance. Previous studies showed that Na^+ exclusion ability in root mature zone in wheat was mainly mediated by SOS1-like Na^+/H^+ antiporters expressed preferentially in the root apex. The relative contribution of this trait to the overall salt tolerance in wheat and barley remains, however, to be quantified. In this work, the ability of root cells to extrude Na^+ from cytosol to apoplast was studied by measuring the extent of Na^+ efflux from the root elongation zone in nearly 100 wheat and barley genotypes, using the MIFE technique. The measured Na^+ efflux was sensitive to amiloride (a known inhibitor of Na^+/H^+ exchangers) and correlated (r = 0.36, $P < 0.05$) with the magnitude of H^+ influx in barley root elongation zone implicating the presence of the functional SOS1 exchangers in the root plasma membrane of barley. In wheat, a significantly ($P < 0.01$) higher Na^+ efflux from root elongation zone was found in bread rather than durum wheat, as well as a significant positive correlation (r = 0.41, $P < 0.01$) was found between root Na^+ exclusion ability and the overall salt tolerance in this species. No significant correlation (r = 0.20, $P > 0.05$) was found between root Na^+ exclusion ability and the overall salt tolerance in barley. The electrophysiological results were consistent with the relative expression level of *HvSOS1* in the barley root apex. Taken together this data suggests that Na^+ exclusion from the root apex is an important component of the tolerance mechanism in salt excluders such as wheat but not in the salt includers such as barley.

By using CoroNa Green dye, the Na^+ distribution between the cytosol and vacuole in root cell was studied using confocal laser microscopy technique. The highest overall Na^+ intensity was found in the root elongation zone in both bread and durum wheat, suggesting Na^+ might be used in osmotic adjustment and turgor maintenance to drive root expansion growth. In bread wheat, salinity stress tolerance correlated positively with cell's ability to sequestrate Na^+ into vacuole in the root mature zone but not in the apex. In contrast to wheat, in barley the ability to sequestrate Na^+ into vacuole has been significantly and positively correlated with its overall salt tolerance in the root elongation

zone. The significant and negative correlation between vacuolar Na^+ sequestration and the overall salt tolerance in wheat root elongation zone was interpreted as the evidence that higher vacuolar Na^+ sequestration in salt sensitive wheat varieties is required to compensate its poor root Na^+ exclusion ability. Interestingly, significantly higher cytosolic Na^+ intensity was found in the meristem root cells in salt *tolerant* but *not sensitive* bread wheat species, while no significant difference in vacuolar Na^+ intensity was detected between them. Similar results were also obtained between bread and durum wheat. This suggests that in addition to its role in cell division, root meristem zone also participates in, or executes, a role of the salt sensor, at least in wheat. No significant difference of biomass and relative water content was found between the plants with intact and cut (to remove the root meristem zone) roots under salt stress. Interestingly, significantly higher chlorophyll content as well as F_v/F_m were found in plants with intact rather than cut roots under salt stress, although no significant difference in leaf Na^+ content was observed. The results indicate that the pattern of Na^+ transport to the shoot and its distribution in the leaf cells under salt stress was affected by the removal of the root meristem zone, suggesting a role of the root meristem as a source of some long distance signal travelling to the shoot to facilitate its adaptation to salinity, at least in wheat.

Interestingly, in contrast to strong correlation in the root mature zone, no correlation was found between the cytosolic K^+ retention in barley root elongation zone and its overall salt tolerance. Together with the fact that NaCl-induced K^+ efflux from barley root elongation zone was 2.3 folds higher ($P < 0.01$) than that from mature zone, it suggests that higher magnitude K^+ efflux in root elongation zone might be required to turn on the "metabolic switch" function to convey salt stress signals. In contrast to the depolarization activated KOR channel mediated NaCl-induced K^+ efflux in root mature zone, cytosolic K^+ retention in root elongation zone was mediated mainly by the voltage-independent NSCC channels, showing the channel specificity in regulating cytosolic K^+ retention in different root zones. Although no significant correlation was found between the overall salt tolerance and both cytosolic K^+ retention and Na^+ exclusion in barley root elongation zone, vacuolar Na^+ sequestration was strongly and positively correlated with the overall salt tolerance. However, increased transcripts levels of *HvNHX1* and *HvVP1* by themselves were not sufficient to achieve efficient vacuolar Na^+ sequestration. This points out to the essentiality of post-transcriptional modifications to achieve gene's function. It also indicates the essentiality of preventing Na^+ back leak from vacuole to cytosol by

efficient control of Na^+ permeable slow (SV) and fast (FV) vacuolar channels. Overall, the results showed that mechanisms involved in K^+/Na^+ homeostasis in conferring salinity stress tolerance are regulated in a highly tissue- and cell type- specific manner.

In conclusion, this work has provided a significant conceptual advance in our understanding of tissue-specificity of salt stress signalling and tolerance in cereals. It also emphasised the importance and a requirement to study salinity tolerance mechanisms at the tissue specific, in addition to traditional whole-plant phenotyping. One of the most significant discoveries was demonstration that mesophyll K^+ retention is a very significant (and previously overlooked) component of salinity tolerance, in both wheat and barley. Targeting the QTL of mesophyll K^+ retention ability and pyramiding it at the top of other salinity tolerance mechanisms might be a promising way for breeding robust salt tolerant genotypes. Also essential is the demonstration that root Na^+ exclusion from uptake is important for wheat but not for barley. Another important finding is that, in a contrast to the root mature zone, cytosolic K^+ retention in barley root elongation zone is not correlated with its overall salt tolerance and is mediated by different ionic mechanisms. These findings require a major rethinking of breeding strategies for these two species. The future work should be focused on the root meristem and its role in salt tress sensing and long-distance signalling. It will be also essential to reveal the relative contribution of various components (e.g. activities of NHX1, vacuolar H-PPase, and FV/SV channels) towards vacuolar Na^+ sequestration, and the modes of their regulation, both at transcriptional and post-translational levels.

Chapter 1

General introduction

1.1 Background

Land salinization is a worldwide issue that threatens agricultural production. It is estimated that by 2050 the world population will reach 9.3 billion (FAO 2013), and global food production will need to increase by 70% by that time (Tester and Langridge, 2010). More than 800 million hectares of land is affected by salinity (Munns and Tester, 2008). Since most crops are sensitive to salt, a shortage of food supply can be forseen if no large improvement in crop salt tolerance is achieved. The complexity of plant salt tolerance mechanism is one of the main reasons impeding the improvement of crop salt tolerance. High salinity in soil increases the osmolality, reduces the water avaibility and disrupts the ion balance of crop plants.

Wheat and barley, the major crops today, are amongst the first domesticated species (Ullrich, 2011) and on a global scale are ranked as the second and fifth most important crops in terms of dry matter production (Baik and Ullrich, 2008). Both of them are classified as glycophytes, and their production is strongly affected by soil salinity. Thus, given the extent of land salinization in the world and the fact that in many countries areas used to cultivate wheat and barley overlap with salinity belt (e.g. in Australia), identifying salt tolerant wheat and barley germplasm remains one of highest priorities for breeders. In the light of predicted rapid population growth, improving salinity tolerance in wheat and barley is an urgent task to cope with the possible shortage of food supply in the near future.

Salinity tolerance is a complex physiological trait composed of numerous sub-traits and controlled by multiple regulatory pathways (Shabala and Cuin, 2008). This includes (but is not limited to) osmotic adjustment in root and leaf tissues; Na^+ exclusion from root uptake; control of Na^+ loading into the xylem and its retrieval from the shoot; vacuolar or tissue-specific Na^+ sequestration; cytosolic K^+ retention; and ROS (reactive oxygen species) detoxification/tolerance. The similarity of size of Na^+ and K^+ and thus the competative binding with enzymes by Na^+ has been deemed as one of the main reason for Na^+ toxicity. Until now, most of the studies in wheat and barley has focused on traits related to sodium, such as Na^+ exclusion from uptake, control of xylem Na^+ loading, Na^+

retrieval from the shoot or vacuolar Na^+ sequestration. However, it is not Na^+ but the K^+/Na^+ ratio in the cytosol which is important for plant performance under saline conditions (Maathuis and Amtmann, 1999; Hauser and Horie, 2010).

In recent years, the ability of the root mature zone to retain K^+ in cytosol for its overall salt tolerance has emerged in many species as an important mechanism, e.g. barley (Chen et al., 2005, 2007c), wheat (Cuin et al., 2008), lucern (Smethurst et al., 2008), and poplar (Sun et al., 2009). However, all the measurements were done in root mature zone, whether the importance of K^+ retention in overall salt tolerance can be extrapolated to other tissues e.g. leaf mesophyll or root apex has never been considered. Achieving better K^+ retention at the whole plant level under salt stress should be attributed to the regulation of K^+ retention at tissue- and cell- specific level by a complicate network.

When Na^+ enters the root cell it causes a depolarization of the membrane potential, inducing K^+ release from cytosol to apoplast and disruption of cytosolic K^+/Na^+ homeostasis culminating in the generation of ROS. Since the first report of overexpression of AtNHX1 Na^+, K^+/H^+ exchanger improved salt tolerance in Arabidopsis (Apse et al., 1999), expressing *NHX1* to improve salt tolerance was widely reported in many species, e.g. wheat (Xue et al., 2004; Saqib et al., 2005b), rice (Chen et al., 2007a), cotton (He et al., 2007), maize (Yin et al., 2004), tomato (Zhang and Blumwald, 2001), peanut (Banjara et al., 2012), tobacco (Gouiaa et al., 2012), and *Brassica napus* (Zhang et al., 2001) etc. However, recently, it has been found that there is no clear improvement of overall salt tolerance in Arabidopsis (Yang et al., 2009) and barley (Adem et al., 2015) by overexpressing NHX1. Furthermore, most studies are focused on the whole plant level and little attention is paid to the tissue and cell specific role of vacuolar Na^+ sequestration in overall salt tolerance.

In most crops, salinity stress tolerance is correlated with its ability to control accumulation of Na^+ in the shoot or leaves (always termed as sodium exclusion) (Matsushita and Matoh, 1991; Garthwaite et al., 2005; Cuin et al., 2010; Munns et al., 2012). However, most researchers didn't distinguish between Na^+ exclusion from shoot/leaves and Na^+ extrusion from root uptake, and rather used them as synonyms. The reduced Na^+ accumulation in shoot could be achieved multiple mechanisms: 1) reduced rate of the unidirectional Na^+ uptake by plant roots; 2) enhanced root Na^+ extrusion back into rhizosphere; 3) reduced rate of Na^+ loading into the xylem; and 4) enhanced retrieval of Na^+ from the shoot and its recirculation back to roots. So, which of these (very different) mechanisms plays the main role in achieving the reduced Na^+ accumulation in

the shoot, or they might be differentially coordinated at species or even tissue specific level? The actual role of the Na^+ extrusion from root uptake (e.g. SOS1-mediated Na^+ efflux) in overall salt tolerance was also not properly targeted and interpreted, at least at tissue specific level. So far, SOS1 (salt overly sensitive 1) Na^+/H^+ antiporter is the only transporter which has been characterized to mediate Na^+ efflux from cytosol to apoplast in plant cell. Mutation of SOS1 showed increased salt sensitivity in many plants e.g. *Arabidospsis* (Shi et al., 2000), tomato (Olías et al., 2009b), and *Thellungiella halophila* (Oh et al., 2009) etc. Also the increased salt tolerance by overexpression SOS1 was found in *Arabidopsis* (Shi et al., 2003, Yang et al., 2009), tobacco (Yue et al., 2012), and tomato (Olías et al., 2009a).

Basically, the above mentioned three components, i.e. Na^+ extrusion from the cytosol to apoplast, vacuolar Na^+ sequestration, and K^+ retention in cytosol, play the main role in controlling K^+/Na^+ homeostasis. However, most of our knowledge about these mechanisms is obtained from the whole plant level, its physiological role in overall salt tolerance at the tissue and cell specific level is rarely studied. Also, the role of leaf tissue specific salinity stress tolerance in the overall salt tolerance in wheat and barley is still obscure.

1.2 Outline of the chapters

The thesis contains 11 chapters and one appendix. Besides the general introduction (chapter 1), literature review (chapter 2), and general discussion (chapter 11), it covers aspects of the role of mesophyll K^+ retention (chapter 3, 4, 5, and appnedix 2), leaf tissue specific salt tolerance (chapter 6), tissue specific vacuolar Na^+ sequestration in root cells (chapter 7, 8, and 9), and root Na^+ extrusion (chapter 8, and 9) in overall salt tolerance in nearly 50 wheat and 50 barley varieties. A brief summary of the main content in each chapter of this thesis is shown below.

Chapter 1 contains a brief introduction of the background and the structure and aims of the present thesis are introduced.

Chapter 2 provides the general background information about how plants maintain the K^+/Na^+ ratio under salt stress. The mechanisms of plants to alleviate Na^+ toxicity and the importance of mesophyll K^+ retention in plant salt tolerance, and also the molecular identity of the ion channels and transporters involved.

Chapter 3 describes the methodological setup of measuring the ability of leaf mesophyll to retain potassium under salt stress via MIFE technique and links it with the possibility of screening salinity tolerance in wheat and barley.

Chapter 4 describes aspects of using MIFE technique to measure NaCl-induced K^+ efflux to screen overall salt tolerance in barley genotypes, and suggests that in salt-sensitive genotypes, increased H^+ extrusion may be needed for charge balancing the activity and also provide the driving force for the high affinity HAK/KUP K^+ transporters required to restore cytosolic K^+ homeostasis.

Chapter 5 describes how the ability of leaf mesophyll to retain K^+ is strongly and positively correlated with overall salt tolerance in wheat, and that bread wheat possesses better mesophyll K^+ retention ability than durum wheat. It also reveals that in contrast to the root mature zone, it is not voltage-gated KOR channels but the non-selective cation channels that played a major role in mediating NaCl-induced K^+ efflux in wheat mesophyll.

Chapter 6 describes aspects of developing and validating a high-througput assay for leaf tissue specific salinity stress tolerance in wheat and barley. It reveals that in contrast to the strong positive correlation between leaf tissue specific salinity stress tolerance and overall salt tolerance in barley, salt sensitive wheat varieties have better ability to handle Na^+ accumulated in the shoot via leaf tissue specific salinity stress tolerance mechanisms to compensate its poor root Na^+ extrusion ability.

Chapter 7 describes aspects of quantifying Na^+ distribution in different root zones in bread wheat and investigates the tissue-specific role of vacuolar Na^+ sequestration in overall salt tolerance. It reveals that vacuolar Na^+ sequestration ability is positively correlated with salinity stress tolerance in the root mature zone but not in the root apex, and the root meristem zone might execute or participate in the role of the "salt sensor".

Chapter 8 describes aspects of cell-specific regulation of ionic homeostasis in the context of salinity stress tolerance using the root elongation zone as a case study, and reveals the physiological and molecular idenetity of mechanisms in controlling K^+/Na^+ homeostasis.

Chapter 9 describes aspects of the tissue specific role of vacuolar Na^+ sequestration and Na^+ extrusion in overall salt tolerance in bread and durum wheat, and the effect of removing the root meristem zone on Na^+ distribution in leaf mesophyll cells of wheat

under salt stress. It reveals that bread wheat showed significantly higher vacuolar Na^+ sequestration ability than durum wheat only in the root mature zone, and that removal of the root meristem zone affects the Na^+ distribution in leaf cells.

Chapter 10 is a chapter of general discussion and includes practical recommendations for improving crop salt tolerance.

Appendix 1 describes a high throughput protocol for using MIFE technique to screen mesophyll K^+ retention ability for crop breeding for salinity tolerance

Appendix 2 describes a series of pharmacological experiments to investigate the role of ROS in regulating NaCl-induced K^+ efflux in wheat leaf mesophyll and reveals that chloroplast-generated ROS dominates NaCl-induced K^+ efflux in wheat leaf mesophyll.

1.3 Aims of the research

The aims of this thesis were five-fold:

1) to investigate the role of mesophyll K^+ retention in overall salt tolerance in wheat and barley, and understand the possible difference of identity of channels mediating NaCl-induced K^+ efflux in leaf mesophyll cell in wheat and barley;
2) to investigate the role of leaf tissue tolerance in overall salt tolerance in wheat and barley and the possible difference in its contribution;
3) to investigate the role of the tissue specific Na^+ distribution in cytosol and vacuole in root zones in overall salt tolerance in wheat and barley;
4) to investigate the role of Na^+ extrusion in the root elongation zone in overall salt tolerance in wheat and barley;
5) to develop rapid and reliable screening methods of evaluation some key physiological traits conferring salinity stress tolerance to be used in breeding programs.

Chapter 2

Literature Review

2.1 Introduction

According to their ability to tolerate saline environments, plant species are classified as glycophytes (all cultivated species; mostly salt sensitive) and halophytes (naturally salt tolerant plants). Many glycophytes are particularly intolerant of salt, with their growth being inhibited by NaCl concentrations as low as 25−50 mM (Haro et al., 1993). Sodium salts dominate in many saline soils of the world (Rengasamy, 2006; Tavakkoli et al., 2010) and an ECe of 4 dS/m (equivalent to 40 mM NaCl) is considered as a threshold of salinity (Munns and Tester, 2008). It is estimated that the progressive increase in soil salinization may result in a loss of about 30% of the arable land within the next 25 years (Wang et al., 2003). To meet the projected demand by feeding 9.3 billion people by 2050, global agricultural production must be increased by 60% from its 2005–2007 levels (FAO report 2013).

Salinity tolerance is a complex, polygenic trait (Flowers, 2004; Zhu, 2000) that is affected by not only physiological mechanisms but also environmental factors. Also, both additive and non-additive effects contribute to its inheritance (Koyama et al., 2001; Dehdari et al., 2007). Therefore, understanding the mechanisms underlying plant salt tolerance would benefit in breeding robust salt tolerant species and thus would provide a feasible way to mittigate the possible food shortage in future.

Na^+ is classified as a beneficial element and as such is not essential for plants, with a possible exception of some C4 species where small amounts of Na^+ in the soil have beneficial effects on plant photosynthetic performance (Kronzucker et al., 2013). Higher Na^+ levels accumulated in the cytosol cause Na^+ toxicity in crops (Luan et al., 2009). Accumulation of high Na^+ in cytosol not only can disrupt various enzymatic processes (Tester and Davenport et al., 2003), but also put an energetic burden on the cell due to the requirement of organic solutes synthesis to compensate for the extrusion of Na^+ for osmotic adjustment (Raven, 1995; Munns and Tester, 2008). Also, the similarity of the hydrated ionic radii of Na^+ and K^+ is one of the main reasons for Na^+ toxicity in plants under salt stress (Blumwald 2000). At the cellular level, many metabolic reactions are catalysed by K^+ with at least 80 enzymes requiring K^+ (Evans and Sorger, 1966; Suelter,

1970). Protein synthesis also requires high potassium concentration (Anschutz et al., 2014; Shabala and Pottosin, 2014). Therefore, a proper ratio between intracellular levels of K^+ and Na^+ is considered to be of a fundamental importance to plant function and growth (Maathuis and Amtmann, 1999).

K^+/Na^+ homeostasis has been long regarded as a key determinant of plant salinity stress tolerance (Yamaguchi and Blumwald, 2005; Chen et al., 2007b, d; Takeda and Matsuoka, 2008; Luan et al., 2009; Olías et al., 2009b; Cuin et al., 2012). In rice, the strongest yield reduction was found in cultivars with the lowest K^+/Na^+ ratio (Asch et al., 2000). K^+ concentration in plant cell cytosol was regarded as ~100 mM (Wang and Wu, 2013). Under saline condition, Na^+ concentration can be elevated from a few mM (non-saline condition) to 30-100 mM in plant cell cytosol (Munns and Tester, 2008; Kronzucker and Britto, 2011). Thus the cytosolic K^+/Na^+ ratio in plant cells is many folds higher than in saline external media like seawater where the K^+/Na^+ ratio is 0.02 (10.4 mM K^+ and 480 mM Na^+, Page and Cera, 2006).

The present review is predominantly focused on the ability of higher plants to alleviate Na^+ toxicity under salinity stress. As such, the focus is on the mechanisms and the molecular identity of transporters involved in Na^+ uptake and sequestration within plant tissues. The physiological and molecular mechanisms behind regulation of K^+ acquisition and maintenance of K^+ homeostasis under salt stress are covered in details in the experimental chapters.

2.2 Regulation of Na^+ transport in plants

2.2.1 Control of Na^+ uptake by the root

Na^+ efflux from the cell is an active process under the presence of elevated levels of Na^+ outside the cell (Apse and Blumwald, 2007). The ion exchange activity between Na^+ influx into plant cells (through ion channels e.g. high affinity K^+ transporter HKT and non-selective cation channels (NSCC)) and Na^+ efflux (through a Na^+/H^+ transporter (e.g. SOS1)) refers to the net Na^+ accumulation in plant cells under salt stress. Until now, SOS1 is the only transporter which has been characterized in Na^+ extrusion from cytosol to the apoplast. GUS analysis showed that SOS1 was mainly expressed in the root apex in *Arabidopsis* (Shi et al., 2002).

It is widely accepted that Na^+ extrusion exerts an important role to protect plants under salinity stress. Whether this is a trait related to root or shoot, however, has remained unclarified. Most studies of the importance of Na^+ extrusion in salt tolerance are based on

shoot/leaf Na^+ content or even at whole plant level (Al-Karaki 2000; Byrt et al., 2007; Møller and Tester 2007; James et al., 2011; Munns et al., 2012; Roy et al., 2013). Our knowledge of the role of root Na^+ extrusion in overall salt tolerance at the physiological level is still rare, especially at tissue or cell type specific level. The discussion of the importance of root Na^+ extrusion in overall salt tolerance in plants can be found in Chapters 9 and 10.

2.2.2 Na^+ sequestration in vacuole

Na^+ sequestration in the vacuole is mediated by Na^+/H^+ antiporters and is regarded as a common mechanism in plants (Apse et al., 1999; Mansour et al., 2003; Rahnama et al., 2011). Compartmentation of Na^+ in the vacuole prevents elevation of cytoplasmic Na^+, raises the cytosolic K^+/Na^+ ratio and contributes to the vacuolar osmotic potential (Maathuis and Amtmann, 1999). As described early, overexpression of a gene NHX1 responsible for Na^+, K^+/H^+ exchanger improves salt tolerance in many species. In barley, tolerant genotypes were shown accumulating significantly more Na^+ in their excised leaves compared with sensitive genotypes suggesting that the activity of the Na^+/H^+ tonoplast exchanger should be much higher in salt tolerant genotypes than sensitive genotypes (Shabala et al., 2010). This is in accordance with the root depletion experiment showing that Na^+ concentration (based on the root FW) remaining in the growth solution is about 40% less in salt tolerant than sensitive varieties (Chen et al., 2007b). Significantly higher vacuolar Na^+ intensity in root mature zone cells was shown in salt tolerant compared to sensitive bread wheat varieties (Cuin et al., 2011). Also, the expression level of *AtNHX1* was significantly increased in leaves, particularly in older leaves of *Arabidopsis,* under salt stress (Shi and Zhu, 2002a). In addition, most of these studies are in shoots, less attention was paid to the role of root vacuolar Na^+ sequestration in overall salt tolerance, especially at the tissue and cell type specific level. This is also one of the research targets of the present thesis.

2.2.3 Control of xylem Na^+ loading and unloading

Ions are absorbed by roots and then transferred to shoots through xylem loading. Control of xylem Na^+ loading is important for plant salt tolerance. Xylem Na^+ loading can be achieved by plasma membrane located SOS1 Na^+/H^+ antiporter (Apse and Blumwald, 2007; Shabala, 2013; Maathuis, 2014), CCC co-transporter (Colmenero-Flores et al., 2007), or SKOR channel (if xylem loading of Na^+ is passive) (Wegner and de Boer,

1997). Shi et al. (2002) reported that SOS1 Na^+/H^+ antiporter is critical for controlling long-distance Na^+ transport from root to shoot in *Arabidopsis*, and suggested that SOS1 may function in xylem Na^+ loading under mild salt stress. Overexpression of SbSOS1 enhances xylem Na^+ loading and confers salt tolerance in transgenic tobacco (Yadav et al., 2012). Recently, Zhu et al. (2015) showed that with down-regulation of SOS1-like Na^+/H^+ antiporter in *Nax* lines there was a reduction in overall net xylem Na^+ loading and accumulation in the shoot and improved salt tolerance. GUS expression analysis showed that CCC co-transporter is preferentially expressed at the xylem/symplast boundary, suggesting its role in xylem Na^+ loading (Colmenero-Flores et al., 2007).

Besides loading Na^+ into xylem, another important process regarding Na^+ transport in xylem is Na^+ unloading from xylem. AtHKT1 transporter was located on the plasma membrane in xylem parenchyma cells in leaves and induced Na^+ unloading from xylem vessels to xylem parenchyma cells (Sunarpi et al., 2005). TmHKT7 transporter was associated with *Nax1* loci and suggested that TmHKT7-A2 could control xylem Na^+ unloading in roots and sheaths (Huang et al., 2006). HKT1;5 (HKT8) is strongly associated with *Nax2* loci in durum wheat and its homeologous loci *Kna1* in bread wheat removes Na^+ from xylem in roots and leads to a high K^+/Na^+ ratio in leaves (Byrt et al., 2007). For more information about the importance of the HKT1 family of Na^+ transporters in Na^+ unloading from xylem refer to Huang et al. (2008).

2.2.4 Na^+ recirculation from shoot to root via phloem

Many studies have suggested that Na^+ recirculation from shoots to roots is an efficient way to protect leaf cells from Na^+ toxicity (Kong et al., 2012). For most salt sensitive plants, the ability to sequester Na^+ into leaf vacuoles is poor and probably the Na^+ recirculation from shoots to roots via the phloem sap is the main mechanism involved in prevention of Na^+ delivery to leaf cells (Berthomieu et al., 2003). It has been suggested that apart from the shoot growth rate (Davenport et al., 2007) the rate of recirculation of Na^+ to the roots via phloem is the final factor affecting Na^+ concentrations in shoots. The recirculation of Na^+ back to the roots via phloem and its role in overall salt tolerance has been reported for several species, including lupin, *Trifolium alexandrinum*, sweet pepper, and maize (Tester and Davenport, 2003). In rice, SKC1, encoding a member of HKT-type transporter, is hypothesised to be involved in the recirculation of Na^+ by unloading it from the xylem (Ren et al., 2005). The recirculation of Na^+ from shoots to roots may help distribute Na^+ more evenly within the whole plant so that other detoxifying mechanisms,

including sequestration in vacuoles, would become more effective (Ren et al., 2005). In *Arabidopsis*, expression of the *AtHKT1* gene, corresponded to the *sas2* locus (sodium overaccumulation in shoot), was shown to be restricted to the phloem tissues in all organs; and the *AtHKT1* gene was involved in the Na^+ recirculation from shoots to roots, probably by mediating Na^+ loading into the phloem sap in the shoots and unloading in roots (Berthomieu et al., 2003). However, Davenport et al. (2007) showed that in *Arabidopsis* AtHKT1 contributes to the control of both Na^+ accumulation in the roots and retrieval of Na^+ from the xylem while not being involved in root influx or recirculation in the phloem.

2.2.5 Na^+ secretion

Secretion of ions by specialized salt glands is a well-known mechanism for regulating the mineral content of many halophytic plants (Liphschitz et al., 1974). These specialized cells (e.g. bladder cells) are thought to serve as a peripheral salinity and/or water storage organ to improve survival under high salinity or water deficit stress conditions (Agarie et al., 2007). In Rhodes grass, the salt glands can secrete both Na^+ and K^+, but the ability to secrete Na^+ is greater than that of K^+ secretion (Kobayashi et al., 2007). In *Aeluropuse littoralis*, excreted salts from special salt glands which were scattered on the both leaf surfaces were mostly sodium and chloride (Barhoumi et al., 2007). In *Avicennia marina* leaves, the number of salt glands increased with external salt concentrations and rates of salt secretion are enhanced greatly when plants are transferred to increasingly strong saline solutions (Chen et al., 2010). Also, through using epidermal bladder-cell-less mutant of common ice plant (*Mesembryanthemum crystallinum)*, Agarie et al. (2007) showed that epidermal bladder cells contribute to succulence by serving as a water storage reservoir and to salt tolerance by maintaining ion sequestration and homeostasis within photosynthetically active tissues. In quinoa, the Na^+ concentration in leaf sap was significantly higher in both brushed and non-brushed old leaves than young leaves which are taken from the plants after treatments with 400 mM NaCl for 3 weeks (Bonales-Alatorre et al., 2013b). Also, young quinoa leaves rely heavily on epidermal bladder cells for Na^+ sequestration, however, old leaves store Na^+ in mesophyll cell vacuoles as it possesses only a few non-collapsed bladders compared with the numerous and inflated epidermal bladder cells in young leaves (Bonales-Alatorre et al., 2013b).

2.3 Actual Na^+ concentrations in plant cell cytosol: still a mystery

Glycophytes vulnerability to high external Na^+ condition is related to a poor ability to tolerate the increased cytosolic Na^+. However, until now, one basic unresolved issue regarding plant salt tolerance is the actual concentration of Na^+ in cell cytosol. A number of approaches have been used to assess the absolute values for Na^+ concentrations in the cells or cell cytosol including isotope flux analysis such as $^{22}Na^+$ flux analysis (Binzel et al., 1988) and $^{24}Na^+$ (Schulze et al., 2012), Na^+ selective ion microelectrode (Dagostino and Lee, 1982; Carden et al., 2003), X-ray microanalysis (Pitman et al., 1981; Binzel et al., 1988), and others. There is still a controversy regarding the toxic level of cytosolic Na^+ concentration in glycophyte plant cells with one side claiming nearly 30 mM as a threshold of cytosolic Na^+ concentration (Tester and Davenport, 2003; Munns and Tester, 2008) while another suggests a range between 50 and 200 mM (Kronzucker and Britto, 2011). In rice suspension cells, cytosolic Na^+ concentration was found to be about ~5 and ~12 mM in tolerant and sensitive varieties under control condition, respectively (Anil et al., 2007). In Arabidopsis root hairs, the measured Na^+ concentration is lower than 65 mM under salt stress (90 mM NaCl, 20 min) (Halperin and Lynch, 2003). Carden et al. (2003) found that in salt stressed barley root cortical cells, the Na^+ concentration in the cytosol ranged between ~10–30 mM. In halophytes, Na^+ concentration in cell cytosol under salt stress ranged between 60 and 200 mM (Flowers et al., 2015).

2.4 Na^+ as a nutrient

Na^+ is one of the most soluble minerals that is easily accessible to plants. Uptake of ubiquitous Na^+ is desirable as a way to build osmotic potential, absorb water and sustain turgor, although excess Na^+ may be toxic (Pardo and Quintero, 2002). Plants use various ways to control excessive intracellular levels of Na^+. The existence of an ATP-driven Na^+ transport mediated by a Na^+-ATPase at the plasma membrane has been showed in lower plants e.g. marine algae *Heterosigma akashiwo* (Wada et al., 1989; Shono et al., 2001) and moss *Physcomitrella patens* (Lunde et al., 2007).

Na^+ ions are taken up into cells against the electrochemical gradient via several pathways, such as non-selective cation channels and the sodium transporter HKT1 (Ward et al., 2003). Under K^+ starvation, moderate amounts of Na^+ can promote plant growth, and a possible explanation for this is the fact that Na^+ may replace K^+ in its osmotic function in the vacuole and thereby make available more potassium to the cytosol (Mäser et al., 2002a; Rodríguez-Navarro and Rubio, 2006). Low to moderate Na^+ concentrations are commonly found to be a benign and even beneficial element, which can stimulate

growth of many plant species, particularly when plants are K^+-deprived (Schulze et al., 2012). For more information about the dual role of Na^+ in a plant's life cycle, please refer to the recent review by Kronzucker et al. (2013).

Evolution would not allow plants to do some futile work and thus to waste the cherished energy from photosynthesis. A narrow focus on the toxic effects of sodium may be impedeing the understanding of the complexity of plant salt tolerance. Halophytes are naturally salt (Na^+) tolerant plants (Flowers and Colmer, 2008; Shabala 2013). The growth of halophyte *Salicornia europaea* was stimulated under 200 mM NaCl compared to the non-saline condition (Lv et al., 2012). Thus, learning from halophytes about its mechanisms to utilize Na^+ as a nutrient or prevent Na^+ accumulation in cell cytosol has been regarded as a useful strategy to improve salt tolerance in glycophytes (Flowers and Colmer, 2008; Flowers et al., 2010; Ruan et al., 2010; Shabala, 2013; Shabala et al., 2014).

2.5 Molecular identity of channels and transporters mediating Na^+ transport and sequestration

Na^+ transport from saline soil to the shoot can be divided into several steps: (1) initial influx of Na^+ into root cells; (2) Na^+ efflux from root cells to the external solution; (3) Na^+ transfer into the xylem for transport to the shoot; (4) removal of Na^+ from the xylem in the shoot, before entry into leaf blades; and (5) recirculation of Na^+ from leaf blades to other tissues including the roots (Davenport et al., 2005). Furthermore, other type of cells and cell compartments such as the apoplast and endodermis are also involved in Na^+ transport process. Here, I briefly review some channels and transporters involved in Na^+ transport and the most recent model about the channels and transporters involved in Na^+ uptake, efflux, and distribution is shown in Fig. 1.

2.5.1 SOS1, Na^+/H^+ antiporter

SOS1 (salt overly sensitive) Na^+/H^+ antiporter is responsible for Na^+ extrusion in plant cells (Shi et al., 2000, Shabala et al., 2005). AtSOS1 belongs to CPA1 (monovalent cation/proton antiporters) family, located on plasma membrane (Gierth and Mäser, 2007). The SOS1 gene was mapped to chromosome 2 at 29.5 ± 6.1 centimorgans (Wu et al., 1996). SOS2 and SOS3, genes found in the same root-bending screen, regulate SOS1 activity. SOS3 is a myristoylated calcium binding protein (CBL4, Luan et al. 2009) that

recruits SOS2 to the plasma membrane. SOS2, a serine/threonine protein kinase (CIPK24, Luan et al., 2009), activates SOS1 by phosphorylation and dramatically increases Na^+/H^+ exchange activity in isolated plasma membrane vesicles (Apse and Blumwald, 2007). The AtSOS2/3 signalling complex has also been suggested to be involved in the regulation of other pathways and proteins that are related to salt stress, including ABA synthesis and the transporters AtNHX1, AtAKT1 and AtHKT1 (Kronzucker and Britto, 2011). AtSOS4 is involved in pyridoxal phosphate (vitamin B6) synthesis and root hair development (Shi and Zhu, 2002b), while AtSOS5 is probably a cell-surface proteoglycan essential for cell expansion and for normal root growth under saline conditions (Shi et al., 2003).

2.5.2 HKT transporters

HKT transporters are members of a large superfamily found in plants, bacteria and fungi (Zhang et al. 2010). HKT transporters in plants have been classified into two subclasses: HKT1;X and HKT2;X. The division of the family into two major branches is associated with a glycine/serine substitution of a residue predicted to be in the first pore loop of the protein (Platten et al., 2006). All members of subfamily 1 have a serine at this position (for the motif S-G-G-G) and show preferred Na^+ conductance, whereas members of subfamily 2 (except for the revertant OsHKT2;1 in which glycine has reverted to serine) have a glycine and show superior K^+ conductance (for the motif G-G-G-G) (Platten et al., 2006; Kronzucker and Britto, 2011). The main role of HKT1;1 until recently was believed to be in regulating Na^+ distribution between root and shoot rather than in mediating primary Na^+ entry into roots. Despite the better K^+ conductance members of the HKT2 family have been clearly shown to be involved in primary Na^+ uptake in roots especially OsHKT2;1 in *Japonica* rice (Kronzucker and Britto, 2011). Also, it is known now that the quantitative trait loci *Nax1* and *Nax2* in durum wheat are linked to HKT1;4 and HKT1;5 transporters, respectively (James et al., 2011), as well as *Kna1* in bread wheat being linked to HKT1;5 transporter (Byrt et al., 2007).

2.5.3 NHX

The intracellular NHX transporters compose the subclass one of the cation/proton antiporter family (putatively a very large number in plants). It plays a crucial role in not only Na^+ accumulation in vacuoles but pH regulation and K^+ homeostasis, regulating processes from vesicle trafficking and cell expansion to plant development (Rodríguez-Rosales et al., 2009). Most members of this family have been identified as Na^+/H^+

antiporters (Wang and Wu, 2013) but a few are K^+/H^+ antiporters. Most of the NHX family members are located on the tonoplast (e.g. AtNHX1, AtNHX2, AtNHX3, AtNHX4, ItNHX1, ItNHX2, and OsNHX1); others are located on the endomembrane system (e.g. AtNHX5, AtNHX6, and LeNHX2), and to date only AtNHX8 is located on the plasma membrane (Gierth and Mäser, 2007). As vacuolar Na^+ sequestration has been considered to be an important determinant to plant salinity tolerance, studies focusing on Na^+/H^+ antiporter and its pathway/network could provide insights to the understanding of plant salinity tolerance.

2.5.4 NSCC channels

Electrophysiological studies suggest that Na^+ influx across the plasma membrane occurs via NSCC/VIC in root cortical cells (Demidchik and Tester, 2002; Apse and Blumwald, 2007; Zhang et al., 2010). Indeed, Na^+ permeability of VI-NSCC has been demonstrated in a range of tissues and species and there is now substantial evidence that root Na^+ influx is to a large extent catalysed by VI-NSCC (Demidchik and Maathuis, 2007). In a seminal study on *Arabidopsis thaliana*, different cation permeabilities relative to Na^+ were: K^+ (1.49) > NH_4^+ (1.24) > Rb^+ (1.15) > Cs^+ (1.10) > Na^+ (1.00) > Li^+ (0.73) > TEA (0.47); in wheat, the series was: NH_4^+ (2.06) > Rb^+ (1.38) > K^+ (1.23) > Cs^+ (1.18) > Na^+ (1.00) > Li^+ (0.83) > TEA (0.20). In rye root, the series were: K^+ (1.36) = Rb^+ (1.36) > Cs^+ (1.17) > Na^+ (1.00) > Li^+ (0.97) > TEA (0.41) (Kronzucker and Britto, 2011). Among different VI-NSCC such as CNGC (cyclic nucleotide gated NSCC), amino acid gated NSCC, and ROS-NSCC, CNGC has attracted more attention than others. Maathuis and Sanders (2001) showed that cyclic nucleotides regulated VIC channels showed no selectivity among monovalent cations in *Arabidopsis* root cells.

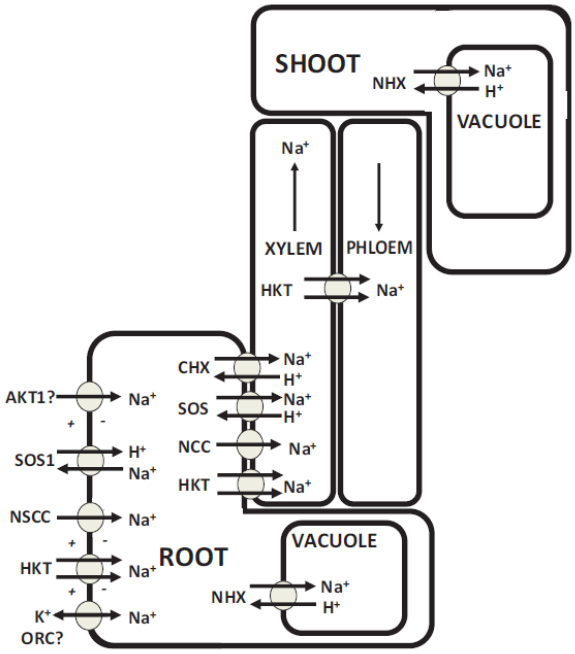


Fig. 1 The most recent model about the transport proteins involved in Na^+ uptake, efflux, and distribution. The model is taken from Maathuis 2014, J Exp Bot 65: 849-858.

2.6 K^+ homeostasis in plant salt tolerance: K^+ concentration in cell compartments

Many approaches have been used to detect the cytosolic K^+ concentration in plant cells, e.g. $^{42}K^+$ flux analysis (Hajibagheri et al., 1988; Schulze et al., 2012), K^+ selective ion microelectrode (Trębacz et al., 1994; Walker et al., 1995; Carden et al. 2003) or longitudinal ion profiles (Jeschke and Stelter, 1976, Hajibagheri et al., 1988), X-ray microanalysis (Pitman et al., 1981; Hajibagheri et al., 1988). The optimal concentration of cytosolic K^+ for enzyme activation in glycophytes was previously regarded as being around 150 mM (Leigh and Wyn Jones, 1984; Chen et al., 2005; Cuin et al., 2008) or between 100-200 mM (Pardo and Quintero, 2002; Luan et al., 2009), but it is now generally accepted to be slightly lower at around 100 mM (Dreyer and Uozumi, 2011; Wang and Wu, 2013). A big variation of cytolplasmic K^+ concentration was found in halophytes, ranging from 32 mM in *Spartina townsendii* (Koyro and Stelzer, 1988) to 225 mM in *Atriplex amnicola* (Jeschke et al., 1986).

K^+ concentration in chloroplast was found to be around 200 mM in spinach grown under non-saline conditions (Robinson et al., 1983; Robinson and Downton, 1984), which dropped to ~120 mM in plants grown under saline condition (Robinson et al., 1983; Robinson and Downton, 1984). Choroplast K^+ concentration varied significantly in halophytes under non-saline condition, e.g. 232 mM in *Suaeda australis* (Robinson and Downton, 1985), 45 mM in *Suaeda maritima* (Harvey and Flowers, 1978). Choroplast K^+

concentration in *Suaeda australis* and *Suaeda maritima* dropped to 73 (Robinson and Downton, 1985) and ~ 29 mM (Harvey et al., 1981; Hajibagheri et al., 1984) under salt stress, respectively.

Vacuolar K^+ concentration is commonly within the range of 20 to 200 mM, depending on the potassium availability and tissue types (Ashley et al., 2006). In K^+ replete barley vacuolar K^+ concentration in leaf epidermis and mesophyll cell was around 200 mM and root epidermis and cortex was around 150 mM (Leigh, 2001). Under salt stress vacuolar K^+ concentration in both barley and durum wheat generally ranged from 40–100 mM in mesophyll cells and 10–50 mM in epidermal cells (James et al., 2006b). Apoplastic K^+ concentration may vary between 10 and 200 or even reach up to 500 mM (Wang et al., 2013). At present, we still lack knowledge about the K^+ concentration in other plant cell organelles e.g. mitochondria, ER (endoplasmic reticulum), and golgi. The K^+ homeostasis in these organelles might be an interesting target for better understanding the effect of salt stress in regulation of plant metabolism and protein transport at the whole cell level in future.

2.7 The importance of K^+ retention ability in plant salt tolerance

K^+ is recognized as a rate-limiting factor for crop yield and quality, and is the major cationic inorganic nutrient in non-hylophytes (Maathuis et al., 1997; Maathuis and Amtmann, 1999; Shabala, 2003; Pettigrew, 2008; Dreyer and Uozumi, 2011), comprising up to 10% of dry matter (Britto and Kronzucker, 2008; Véry and Sentenac, 2003). The function of K^+ in plant life cycle includes 1) acting as a dominant counterion to balance the negatively charged proteins and nucleic acids, 2) enzyme activation, 3) stabilization of protein synthesis, 4) formation of membrane potential, 5) osmoticum for turgor pressure, 6) maintenance of cytosolic pH homeostasis etc. (Shabala, 2003; Dreyer and Uozumi, 2011; Wu et al., 2013). Not surprisingly, K^+ plays an important role in plant response to not only biotic stress such as disease and pests, but also to abiotic stresses, e.g. drought, salinity, cold and frost, and waterlogging (Cakmak, 2005; Wang et al., 2013). It is also suggested that K^+ loss is a critical step in initiation of plant programmed cell death (PCD) (Peters and Chin, 2007). As mentioned above, K^+ is essential for many metabolic reactions at the cellular level. Salinity stress induces chronic K^+ deficiency in plants (Pardo and Quintero, 2002), and the capacity of plants to counteract salinity stress strongly depends on K^+ availability (Maathuis and Amtmann, 1999). K^+ deficiency leads to growth arrest, impaired phloem transport of sucrose, redistribution of K^+ from mature

to developing tissues, reduced photosynthesis, reduced water content, and replacement of K^+ with an alternative osmoticum (Tsay et al., 2001).

In contrasting wheat varieties, the mean K^+ efflux after salt application was found to be significantly different (2 fold) and strongly and negatively correlated with plant yield at harvest (Cuin et al., 2008). In salt-stressed barley roots, salt tolerant varieties showed 3-fold higher ability to retain K^+ in the roots by minimizing NaCl−induced K^+ efflux from epidermal cells, and also solution depletion experiments showed that salt tolerant genotypes were able to loose approximately 80% less K^+ than salt sensitive barley genotypes (Chen et al., 2007b). Moreover, by using the contrasting varieties of lucerne, Smethurst et al. (2008) found that the tolerant varieties had better K^+ retention ability in roots than the sensitive varieties. Similar results were also reported for poplar (Sun et al., 2009). Taken together, these results suggest that the ability of root cells to maintain K^+ in cytosol under salt stress is important for the plants overall salt tolerance.

2.8 Molecular identity of channels and transporters mediating K^+ homeostasis

In most soils, free K^+ levels are below 1 mM so active transport is required to reach the higher concentration of cytosolic K^+ (around 100 mM) in plant cells. Potassium transport across plant membranes is mediated by at least seven major families of cation transporters and the most recent model of channels and transporters involved in K+ uptake, efflux, and distribution is shown in Fig. 2.

Typical dual-affinity mechanisms are known in K^+ uptake in higher plants: high affinity K^+ uptake mechanism and low affinity K^+ uptake mechanism which mediate K^+ transport at low (below 0.2 mM) and relatively high (above 0.3 mM) external K^+ concentration, respectively (Wang and Wu, 2013). Until now, seven channel and transporter families involved in K^+ transport have been well characterized including: (1) voltage-gated shaker-type potassium channels (AKT1, SPIK, KAT1, KAT2, AtKC1, AKT2, GORK); (2) voltage-independent tandem-pore potassium (TPK) channels (TPK1 (KCO1), TPK2 (KCO2), TPK3 (KCO6), TPK4 (KCO4) and TPK5 (KCO5)); (3) voltage-independent Kir-like (K^+ inward rectifier) channel (KCO3); (4) non-selective cation channels (constitutive plasma membrane NSCC, cyclic nucleotide-gated channels (CNGC), slow vacuolar (SV) channel, fast vacuolar (FV) channel, glutamate receptors (GLR)); (5) KUP/HAK/KT transporters (KUP1, KUP2, KUP4, HAK5 etc.); (6) HKT transporters (HKT2 subfamily); (7) K^+/H^+ antiporters (NHX1/NHX2, CHX13, CHX17,

CHX23 etc.). The unique common feature of all K^+ channel subunits identified so far is the presence of a conserved pore region (P domain) which contributes to K^+ conductivity (Czempinski et al., 2002). The highly conserved selectivity filter comprises the characteristic amino acid motif TxGYGD in the pore loops (Dreyer and Uozumi, 2011).

LCT1 transporter is a wheat polypeptide shown by heterologous expression in yeast to mediate low-affinity transport of a wide range of cations (monovalents e.g. Rb^+, Na^+, Ca^{2+}, Cd^{2+}, but not Zn^{2+}) (Schachtman et al., 1997; Diatloff et al., 2006). Furthermore, Colmenero-Flores et al. (2007) showed that *AtCCC* encodes the homologue of the animal bumetanide-sensitive $Na^+/K^+/2Cl^-$ co-transporter. Also, OsCCC1 has been shown to play a significant role in K^+ homeostasis and rice plant development (Kong et al., 2011).

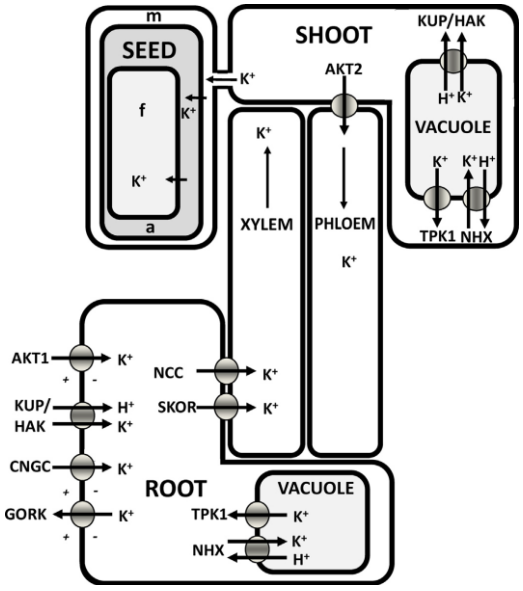


Fig. 2 The most recent model of the transport proteins involved in K^+ uptake, efflux, and distribution. Taken from Ahmad and Maathuis 2014, J Plant Physiol 171: 708-714.

2.9 Conclusion

Due to the complexity of plant salinity tolerance and the multigenic nature of the traits, there was modest progress in elucidating the molecular mechanisms behind plant salinity tolerance. Although plant salt tolerance mechanisms are characterized, our limited understanding of the coordination and networking of these mechanisms (especially at the tissue and cell type specific level) in plant overall salt tolerance compromised and delayed the efforts to breed salt tolerant crops. Also, more work is required to better understand the role of salt tolerance mechanisms (from physiological/molecular level to tissue specific level) in plant salinity tolerance traits e.g. the ability to maintain K^+/Na^+ ratio homeostasis. The latter can be achieved mainly through Na^+ extrusion/sequestration and K^+ retention.

Chapter 3

Ability of leaf mesophyll to retain potassium is essential to confer salinity tolerance in wheat and barley[#]

Abstract

This work investigated the importance of the ability of leaf mesophyll cells to control K^+ flux across the plasma membrane as a trait conferring tissue tolerance mechanism in plants grown under saline conditions. Four wheat (*Triticum aestivum* and *Triticum turgidum*) and four barley (*Hordeum vulgare*) genotypes contrasting in their salinity tolerance were grown under glasshouse conditions. Seven to 10 day-old leaves were excised, and net K^+ and H^+ fluxes were measured from either epidermal or mesophyll cells upon acute 100 mM treatment (mimicking plant failure to restrict Na^+ delivery to the shoot) using non-invasive microelectrode ion flux measuring (the MIFE) system. To enable net ion flux measurements from leaf epidermal cells, removal of epicuticular waxes was trialled with organic solvents. A series of methodological experiments was conducted to test the efficiency of different methods of wax removal, and the impact of experimental procedures on cell viability, in order to optimise the method. A strong positive correlation was found between plants' ability to retain K^+ in salt-treated leaves and their salinity tolerance, in both wheat and especially barley. The observed effects were related to the ionic but not osmotic component of salt stress. Pharmacological experiments have suggested that voltage-gated K^+ permeable channels mediate K^+ retention in leaf mesophyll upon elevated NaCl levels in apoplast. It is concluded that MIFE measurements of NaCl-induced K^+ fluxes from leaf mesophyll may be used as an efficient screening tool for breeding in cereals for salinity tissue tolerance.

Keywords

Potassium retention, salinity tolerance, sodium, membrane depolarization, ion channel, H^+-ATPase

Abbreviations

BSM, basic salt media; BSTFA, N,O-bis-trimethylsilylacetamide; MAS, marker-assisted selection; MIFE, microelectrode ion flux estimation; MP, membrane potential; NSCC, non-selective cation channels; PCD, programmed cell death; PSII, photosystem II; TEA,

[#], This chapter has been published as: Wu H, Shabala L, Barrey K, Zhou M, Shabala S (2013) Physiologia Plantarum 149: 515–527.

tetraethylammonium chloride; TMS, tri-methyl-silyl; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Vanadate, sodium orthovanadate.

3.1 Introduction

Salinity affects over 800 million hectares of agricultural land, which impacts crop production across the globe (Rengasamy, 2010). To match the predicted population growth with food supply salt tolerant crops have to be bred and recommended to growers.

Salinity stress tolerance is a complex polygenic trait that is conferred by multiple physiological mechanisms (Flowers, 2004; Shabala and Cuin, 2008; Munns and Tester, 2008). Because of this, pyramiding physiological traits contributing to salinity tolerance is arguably the only practical way to improve salinity stress tolerance in crops. This also poses a question of what specific trait should be used to identify the source of salt tolerance genes to be used in marker assisted selection (MAS)-based breeding programs. Various agronomical traits such as germination, survival and growth rates; the extent of leaf injury; biomass production (Mano and Takeda, 1997; Eleuch et al., 2008; Zhou et al., 2012; Xu et al., 2012) and whole-plant physiological characteristics such as CO_2 assimilation; chlorophyll content; shoot Na^+ and K^+ content (James et al., 2002; Chen et al., 2005; Munns et al., 2006) have been frequently advocated. To the large extent, the choice has been determined by the convenience (e.g. its cost- and time-efficiency) of the measuring procedure. This is hardly right as the screening technique should target the mechanism directly contributing towards salinity stress tolerance. Striking the right balance is not an easy task.

Until now, researchers have focused predominantly on traits related to sodium, such as Na^+ extrusion from uptake, control of xylem Na^+ loading, Na^+ retrieval from the shoot, or vacuolar Na^+ sequestration (Blumwald et al., 2000; Horie and Schroeder, 2004; Munns and Tester, 2008; Plett et al., 2010; Cuin et al., 2011). However, it is not Na^+ but the K^+/ Na^+ *ratio* in the cytosol which determines plant performance under saline conditions (Maathuis and Amtmann, 1999; Shabala and Cuin, 2008). K^+ is recognised as a rate-limiting factor for crop yield and quality, directly contributing to cell turgor and hence growth (Dreyer and Uozumi, 2011). At the cellular level, maintaining intracellular K^+ homeostasis is essential for enzyme activation, stabilization of protein synthesis, neutralizaiton of negatively charged proteins, formation of membrane potential, and maintenance of cytosolic pH homeostasis (Shabala, 2003; Dreyer and Uozum, 2011). Potassium also plays an important role in controlling apoptosis in animal tissues (Hughes

and Cidlowski, 1999), and a direct causal link between plant ability to control fluxes of K^+ across the plasma membrane and programmed cell death (PCD) was reported for a range of abiotic stress conditions (Shabala et al., 2007b, Demidchik et al., 2010), including salinity.

We have previously reported a strong correlation between plant ability to retain K^+ and salinity tolerance in both barley (Chen et al., 2005; Chen et al., 2007c,d) and wheat (Cuin et al., 2008; Cuin et al., 2012), as well as provided strong evidence for the heritability of this trait (Chen et al., 2007b; Cuin et al., 2012). All these studies were done in roots. However, it is ultimately a plant's ability (or inability) to control the K^+/ Na^+ ratio in photosynthetically active leaf tissues that determines its photosynthetic competence (and hence growth and yield) under saline conditions. Indeed, each of above Na^+-related mechanisms (e.g. extrusion from uptake; restricted loading into xylem; and retrieval from the shoot) has potential limitation and cannot operate indefinitely in a broad range of salinities. Exposure of the plant to longer durations and more severe salt stress increases the chance that one of these mechanisms will fail and high amounts of Na^+ will be delivered to the shoot with a transpiration flow. This will result in significant membrane depolarisation and a massive K^+ leak from leaf mesophyll (Shabala and Cuin, 2008). The consequence of this will be reduced metabolic (e.g. Rubisco; Osaki et al., 1993) activity, reduced cell turgor, impaired Na^+ sequestration in the vacuole (resulting from K^+ requirements for vacuolar PP-ase activity; Davies et al., 1992), and a danger of undertaking PCD resulting from increased activities of proteases and endonucleases at low cytosolic K^+ (Demidchik et al., 2010). Thus, the ability of leaf mesophyll cells to control K^+ flux across the mesophyll plasma membrane may be a very essential component of tissue tolerance mechanisms, contributing to overall plant performance under saline environment. However, to the best of our knowledge, no studies have been conducted so far looking at the extent of genetic variability in this trait. Thus, the aim of this study was two-fold: (1) to determine to what extent salinity tolerance in wheat and barley is related to the ability of mesophyll tissue to retain K^+ when exposed to elevated NaCl levels in the apoplast, and (2) to develop efficient screening protocols allowing rapid assessment of a large number of samples for the above trait.

3.2 Materials and methods

3.2.1 Plant material

Four wheat (*Triticum aestivum* cultivars Kharchia 65 and Baart 46, and *Triticum turgidum* spp. *durum* cultivars Wollaroi and Tamoroi) and four barley (*Hordeum vulgare* cultivars CM 72, Gairdner, TX9425, and Naso Nijo) varieties, contrasting in their salinity tolerance, were used in this study. Among wheats, bread wheat variety Kharchia and durum wheat variety Wollaroi were previously reported as being tolerant, while Baart and Tamaroi were sensitive to salt (Cuin et al. 2008). Among barley varieties, CM72 and TX9425 were deemed tolerant, while Gairdner and Naso Nijo were highly sensitive (Chen et al., 2007c; Xu et al., 2012). All seeds of these varieties were obtained from various sources in the past and multiplied in our laboratory. Plants were grown using University of Tasmania glasshouse facilities essentially as described in Chen et al. (2007d). Two to 3-weeks old plants were used for measurements.

3.2.2 Sample preparation and wax removal trials

Seven to 10 day-old leaves were excised by a razor blade and brought into the laboratory. The presence of epicuticular waxes poses a methodological problem for ion flux measurements (Shabala and Newman, 1999) in cereals, and removing these without altering cell function was required to develop an efficient screening protocol. Leaf cuticular wax removal was trialled with different solvents (either 25% or 96% methanol, or analytical grade hexane), application methods (immersion or rubbing) and duration of application (for the rubbing procedure). The effect of these procedures on efficiency of wax removal, ability to measure K^+ flux and toxicity to the plant was determined, as outlined below.

After wax removal, leaves were immediately and thoroughly rinsed in running distilled water for several minutes. Small leaf segments (approx. 5× 8 mm), were cut and left floating (rubbed surface down) on experimental Basic Salt media (BSM) solution (0.1 mM $CaCl_2$ and 0.5 mM KCl; pH 5.7 non-buffered) overnight in the dark to minimize possible confounding effects of tissue damage on ion fluxes (Shabala and Newman, 1999). Net ion fluxes were then measured from the epidermal leaf surface on which cuticular wax was removed. To measure net fluxes of K^+ and H^+ from leaf mesophyll, a cross-sectional cut was made across the middle part of the leaf blade exposing leaf mesophyll tissue. Cut samples (from 7 to 10 day-old leaves) were then placed onto the surface of BSM solution and kept in the dark until measured.

3.2.3 Wax analysis

To compare the efficiency of wax removal methods, epicuticular wax was extracted from segments of wheat using dichloromethane with docosane (C-22 alkane) as an internal standard (2 mg docosane was diluted in 100 ml dichloromethane). Leaf segments were soaked in 10 ml of solvent for 5 mins with gentle stirring. This solution was decanted, taken to dryness under a stream of N_2, then derivitized with a double TMS (tri-methyl-silyl) method. Briefly, 10 µl of pyridine and 40 µl of BSTFA (N,O-bis-trimethylsilylacetamide) was added, samples were then heated at 80 ºC for 20 min, then taken to dryness. A second smaller volume of pyridine and BSTFA was added; samples were heated at 80 ºC for 15 min, and then dried again. Samples were analysed by combined gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard 5890 Gas chromatograph coupled to a Hewlett-Packard 5970B mass selective detector. One-microliter injections were made in the splitless mode, with an injection temperature of 300 °C and transfer line temperature of 290 °C. The column was a 25 m x 0.32 mm HP1 (0.17 µm film thickness), with a temperature program of 60 °C (held for 1 min) to 300 °C at 10 °C/min, with an 8-min hold time at the final temperature. Mass spectra were collected over the range *m/z* 40 to *m/z* 550 at two scans per second. Total wax was expressed as the ratio of C28 alcohols (the TMS derivatives) to the C22 internal standard.

3.2.4 Non-invasive ion flux measurements

Net K^+ and H^+ fluxes were measured using the non-invasive microelectrode ion flux estimation (MIFE) technique (UTas Innovation Ltd., Hobart, Tasmania) essentially as described previously (Shabala and Shabala, 2002, Tegg et al.; 2005). The theory of MIFE measurements is available elsewhere (Newman, 2001). Briefly, microelectrodes were pulled from borosilicate glass capillaries (GC150-10; Clark Electrochemical instruments, Pangbourne, Berks, UK), dried in the oven at 225 ºC overnight, and then silanized with tributilchlorosilane (catalogue no. 90796; Fluka, Busch, Switzerland). Dried and cooled microelectrodes were backfilled with appropriate solutions (15 mM NaCl + 40 mM KH_2PO_4; pH 6.0 adjusted using NaOH for H^+ or 200 mM KCl for K^+). Then, the tip of the electrodes was filled with commercially available ionophore cocktails (K^+, 60031; H^+, 95297, Fluka, Busch, Switzerland), and prepared microelectrodes were calibrated using a set of appropriate standards before and after use. Only electrodes with a slope of above 50 mV per decade and correlation 0.999 or better were used.

3.2.5 Experimental procedure

Leaf samples were mounted in a Perspex holder and placed into measuring chambers filled with measuring solution (0.1 mM $CaCl_2$ and 0.5 mM KCl). Electrodes were mounted on a 3D-micromanipulator (MMT-5, Narishige, Tokyo, Japan), tips were positioned close together and at 40 μm from the leaf sample. The angle between the tip of electrodes and measured exposed mesophyll cells was set to 30º. During measurements the electrodes were moved by a computer-controlled hydraulic manipulator between two positions, 40 and 110 μm from the leaf surface, in a 12-s square-wave cycle. Net ion fluxes were calculated from recorded electrical potential differences using cylindrical diffusion geometry (Newman, 2001). Under 200 X microscope, the exposed mesophyll cell can be easily distinguished from other cells (to make sure that the measured ion fluxes were dominantly originated from nesophyll cells) and the exposed area of mesophyll cells was calculated and recorded accordingly. Net ion fluxes were measured for 5-10 min under control (BSM) conditions, before 100 mM NaCl was added to the bath followed by another 50 to 60 min of measurements. In some experiments, 170mM mannitol treatment (isotonic to 100mM NaCl) was used of NaCl.

3.2.6 Membrane potential measurements

Conventional KCl–filled Ag/AgCl microelectrodes with tip diameter 0.5 μm were used to measure membrane potential of wheat leaves segments. Measurements were taken from at least four individual plants for each treatment, with 4 to 5 measurements taken from each leaf segment. Membrane potentials were recorded for at least 30 secs after the potential stabilised following cell penetration. Measurements were carried out on epidermal cells treated with the most efficient method of wax removal (as described in the section Results).

3.2.7 Chlorophyll fluorescence

The maximal photochemical efficiency of PSII was estimated by measuring the chlorophyll fluorescence F_v/F_m ratio (Smethurst and Shabala, 2003) of the leaf segments that were subjected to different wax removal methods.

3.2.8 Pharmacology

Further insights into mechanisms underlying leaf ability to retain K^+ upon salinity treatment were obtained by conducted a series of pharmacological experiments using barley variety Gairdner. In these pharmacological experiments, leaf segments were pre-treated with either 20 mM tetraethylamonium chloride (TEA^+; a known blocker of K^+

selective channels), or 1 mM sodium orthovanadate (Na_3VO_4 ; a known inhibitor of P-type H^+ATPase) before being exposed to NaCl treatment. All chemicals are from Sigma, St. Louis, MO.

3.2.9 Statistical analysis

Data were analysed with SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA). All data are given as mean ± SE (n = sample size). All of the replicates are biological replicates. Significance was determined by one-way ANOVA based on Duncan's multiple range test. Significant differences ($P \leq 0.05$) between treatments were denoted by different lower case letters.

3.3 Results

3.3.1 Methodological aspects of ion flux measurements from leaf epidermis and requirement for wax removal

The leaf surface is covered by a cuticle which is composed of a matrix of the biopolymer cutin and waxes of various composition that are usually referred to as epicuticular waxes (Bewick et al., 1993). Epicuticular waxes plays an important role in plant function, including prevention of non-stomatal water loss, defending leaves from bacterial and fungal pathogens, alleviating detrimental effects of abiotic stress such as UV damage, and playing a role in the interaction between plants and insect herbivores (Bewick et al., 1993, Rhee et al., 1998). However, their presence makes the leaf surface highly hydrophobic and almost impermeable to water and ion movement, posing a methodological problem for ion flux measurements (Shabala and Newman, 1999). Both wheat and barley have significant wax deposits (data not shown). When challenged with stress factors, these species displayed little if any ion exchange across the leaf surface when wax was present (Fig. 1A). The 100 mM NaCl treatment caused no detectable K^+ flux signal when measured from untreated leaf epidermis, with epicuticular waxes present (Fig. 1A; closed circles). This may be caused by either waxes restricting Na^+ uptake into the epidermal cells (thus, preventing NaCl-induced plasma membrane depolarization and associated K^+ efflux as reported for other non-cutinised leaf tissues; Shabala, 2000; Shabala et al., 2006), or by preventing K^+ leakage from beneath the epicuticular barrier, or both. This poses a serious methodological issue and prevents studying the effect of salinity on ionic relations in leaves with the microelectrode MIFE technique.

An efficient way to deal with the problem may be to remove wax deposits by some organic solvents, e.g. chloroform, methanol, ethanol, and hexane (Bewick et al., 1993, Shabala and Shabala, 2002; Živanović et al., 2005; Agrawal et al., 2007). The extent of the wax removal will be critically dependent on the concentration of solvent used, and the duration of exposure. While the longer and higher treatments will certainly assure more effective wax removal, leaf intoxication is likely to occur. Thus, the process of removal of epicuticular waxes needs to be finely balanced.

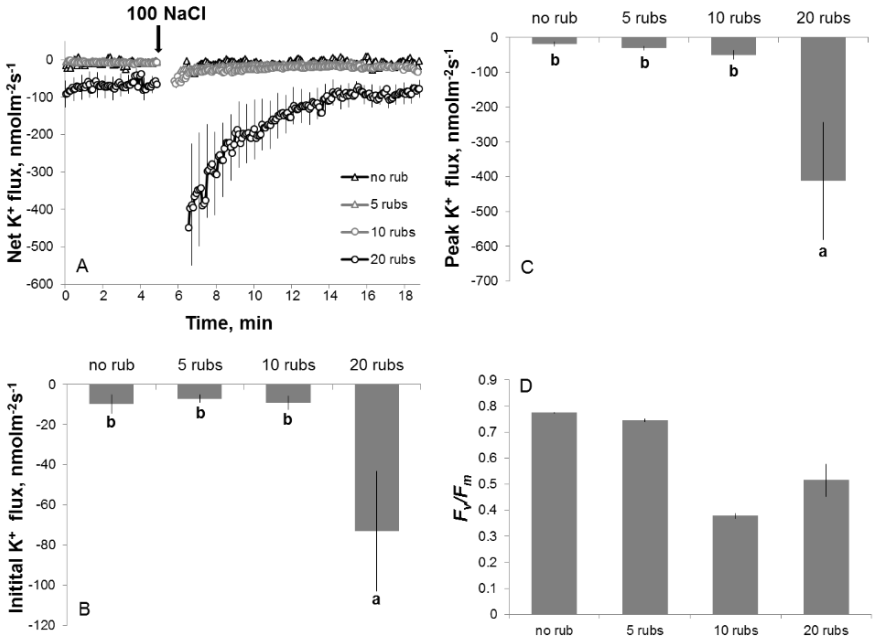


Fig. 1. Ion fluxes measured from leaf surface after removal of cuticular waxes by hexane. Transient net K^+ fluxes (A) and transient net K^+ fluxes; (B) of Kharchia 65 leaf measured after rubbing by hexane for 5, 10, and 20 times; (C) initial (steady-state) K^+ fluxes; (D) peak K^+ fluxes. Means ± SE (n=4-6). Within each treatment different lowcase letters indicate significant differences by Duncan's multiple range test at $p < 0.05$.

We investigated the above problem first by using analytical grade hexane and rubbing the leaf surface with hexane-wetted cotton buds for various number of times (hence, different exposures). Rubbing the leaf surface with hexane 5 or 10 times did not enable measurement of NaCl-induced K^+ fluxes from epidermal cells under salt treatment (Fig. 1A-C). Longer exposures to hexane (20 rubs) make NaCl-induced K^+ flux detectable (Fig. 1A-C), however this treatment also caused significant leaf intoxication as judged by a very significant decline in leaf chlorophyll fluorescence characteristics (F_v/F_m values; Fig. 1D). Thus, it was concluded that hexane was not suitable to be used for efficient wax removal to measure net ion fluxes from leaf epidermal cells without affecting their metabolism.

Methanol was another organic solvent used in an attempt to remove wax from the leaf surface. No response to salt treatment was detected in leaves rubbed five times with 25% methanol, while 10 and 20 rubs resulted in a detectable K^+ efflux, with the flux magnitude being proportional to exposure to methanol (Fig. 2A). When 96% methanol was used, the most efficient treatment was using 10 rubs (Fig. 2B). However, even in this case wax deposits were clearly seen on the leaf surface after rubbing (data not shown), and the amount of wax deposits removed was only approx. 50% (Fig.1C). Leaf immersion in methanol for either 5 or 20 minutes (without rubbing) was less efficient for wax removal than rubbing (Fig. 2B).

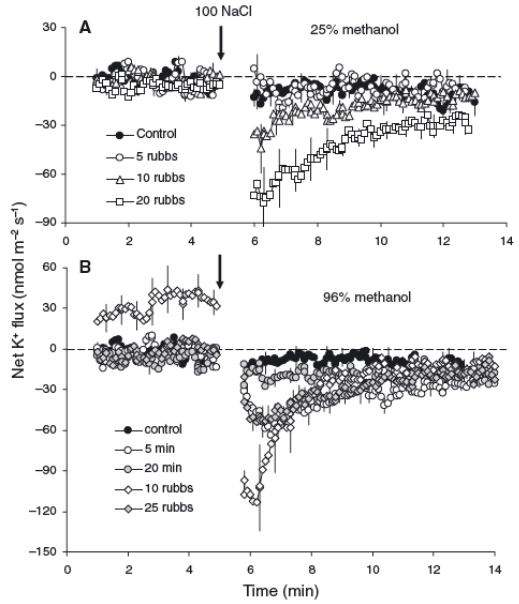


Fig. 2. Ion fluxes measured from leaf surface after removal of cuticular waxes by methanol. (A) kinetics of K^+ flux responses of rubbing different times by 25% methanol; (B) kinetics of K^+ flux responses of rubbing different times by 96% methanol and soaking in 5 and 20 min in 96% methanol. Mean ± SE (n = 5-8).

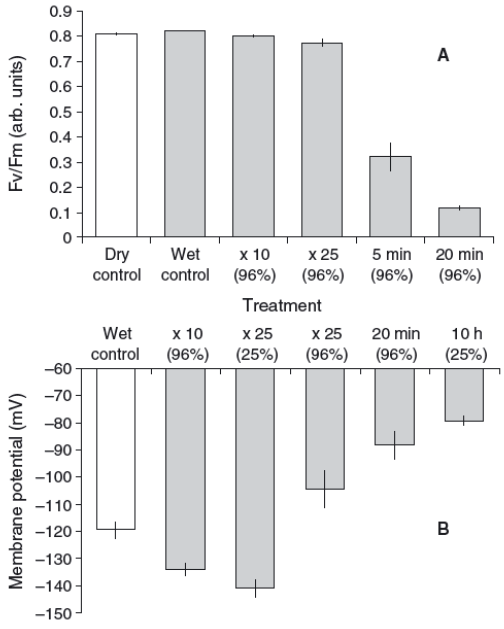


Fig. 3. Assaying leaf viability after removal of cuticular waxes by methanol. (A) chlorophyll fluorescence F_v/F_m values. Mean ± SE (n = 12-15); (B) membrane potential values. Mean ± SE (n = 10-12).

3.3.2 Plant ability to retain K^+ in salt-treated leaves correlates with salinity tolerance

Four wheat cultivars contrasting in salinity tolerance (salt tolerant Kharchia 65 and Wollaroi; salt sensitive Baart 46 and Tamaroi) were studied for their ability to retain K^+ when treated with high (100 mM) concentration of NaCl. In biological terms, this treatment mimics the failure of plants to control xylem Na^+ loading and, therefore, addresses the issue of tissue tolerance in leaves. Net K^+ fluxes were measured from leaf epidermis after removal of cuticular waxes with the optimal method described above (i.e. 10 rubs of 96% methanol).

There were strong correlations between the magnitude of NaCl-induced K^+ flux and salinity tolerance for the three parameters analysed; peak K^+ efflux upon NaCl exposure (Fig. 4A), average K^+ efflux over 15 min (Fig. 4B), and steady-state K^+ efflux at the end of transient (Fig. 4C). K^+ efflux measured from tolerant Kharchia 65 (bread) and Wollaroi (durum) varieties was only 50% of that measured from two sensitive varieties (Fig. 4).

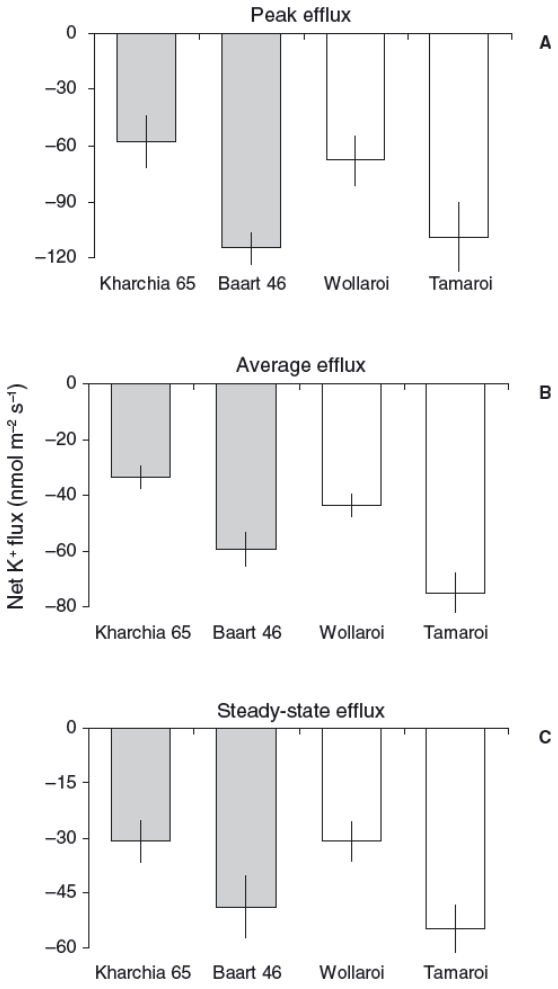


Fig. 4. Net K^+ fluxes measured from the epidermal leaf surface of 4 wheat cultivars contrasting in salinity tolerance. Epidermal waxes were removed by rubbing the leaf surface 10 times by 96% methanol. (A) peak K^+ flux values; (B) total amount of K^+ leaked from the mesophyll tissue over 15 min period; (C) steady- state K^+ flux values; Filled bars – bread wheats (Kharchia 65 – tolerant; Baart 46 – sensitive); open bars – durum wheats (Wollaroi– tolerant; Tamaroi – sensitive). Mean ± SE (n = 5 - 7).

The above efflux of K^+ upon NaCl treatment may originate either directly from the leaf epidermis, from the underlying mesophyll layer, or both. Since mesopyll is the most important metabolically active tissue in plants, its ability to retain K^+ was expected to be more essential to leaf salinity tissue tolerance. Thus, the extent of NaCl-induced K^+ leak from wheat and barley mesophyll was also investigated in direct experiments (Fig. 5, Fig. 6).

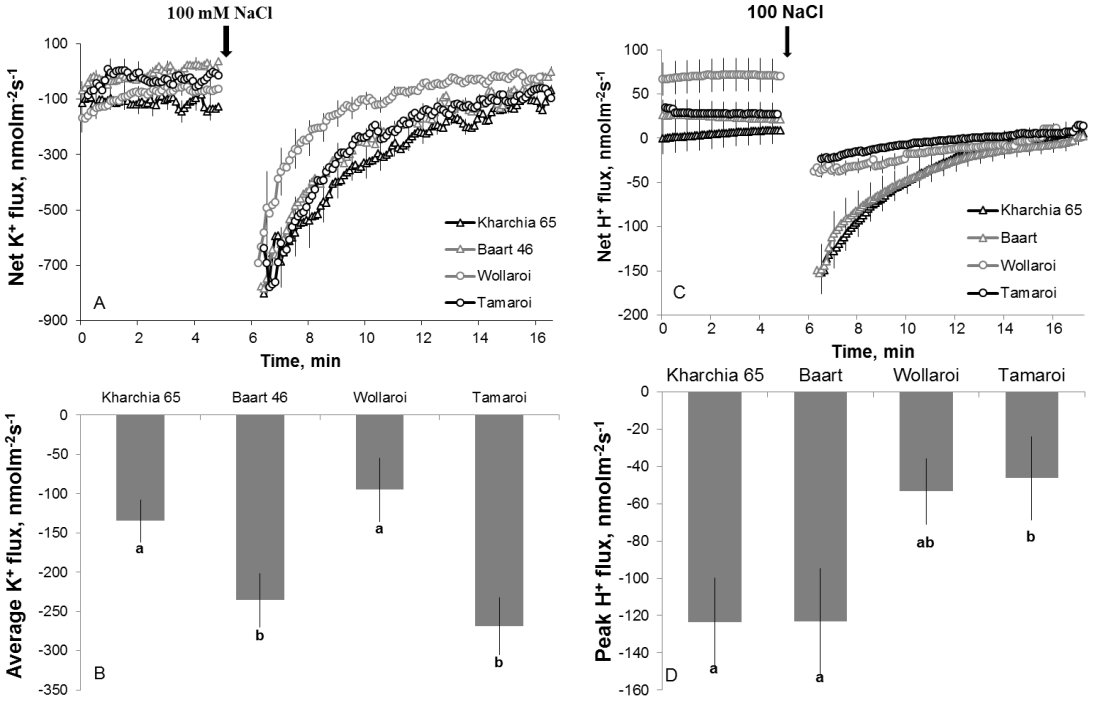


Fig. 5. Net K^+ (A, B) and H^+ (C, D) flux responses measured from the mesophyll tissue of 4 wheat cultivars contrasting in salinity tolerance (as above). To expose mesophyll, the excised leaf (middle part) was cut to segments (roughly 5 × 8 mm) by a sharp blade angularly. (A) kinetics of K^+ flux responses; (B) total amount of K^+ leaked from the mesophyll tissue over 15-min period; (C) kinetics of H^+ flux responses; (D) peak H^+ flux values. Mean ± SE (n = 6-10). Within each treatment different low case letters indicate significant differences by Duncan's multiple range test at $p < 0.05$.

The observed results were consistent with our observations for epidermal tissue. In wheat, the average K^+ efflux from mesophyll (Fig. 5A, B) was 3 to 4-fold higher compared with the flux from epidermal cells (Fig. 4B). This is hardly surprising, given the lack of blocking effect of the cuticle on ion fluxes measured from mesophyll tissue. The qualitative patterns of genetic variability in K^+ retention in mesophyll were generally consistent with epidermal data (Fig. 5B). Similar to data from epidermis, salt-sensitive durum wheat Tamaroi showed a 2-fold higher K^+ leak from its mesophyll, compared with tolerant durum wheat variety Wollaroi (significant at $P < 0.05$; Fig. 5B). The difference between bread wheats was also consistent with epidermal data (Fig. 5B).

Previously, a genetic variability in K^+ retention in barley roots was attributed to better control of membrane potential and higher H^+ pump activity (Chen et al., 2007c). In order to investigate the relationship between K^+ and H^+ response in wheat leaf mesophyll cells under salt stress, NaCl-induced net H^+ flux was also measured. Significantly more H^+ flux was pumped out from mesophyll cells of the two bread wheat varieties, as compared with durum wheat (Fig. 5C, D). This is consistent with the general consensus that durum wheat is generally more sensitive to salt than bread wheat (Munns and Tester, 2008; Cuin et al., 2009).

The strong correlation between the magnitude of NaCl-induced K^+ efflux and salinity tolerance was even more pronounced in barley (Fig. 6A, B). Both peak and steady-state K^+ fluxes were significantly higher in two sensitive varieties (Gairdner and Naso Nijo) compared with two tolerant varieties (CM 72and TX9425). No clear trends were observed for H^+ flux data (Fig. 6C, D).

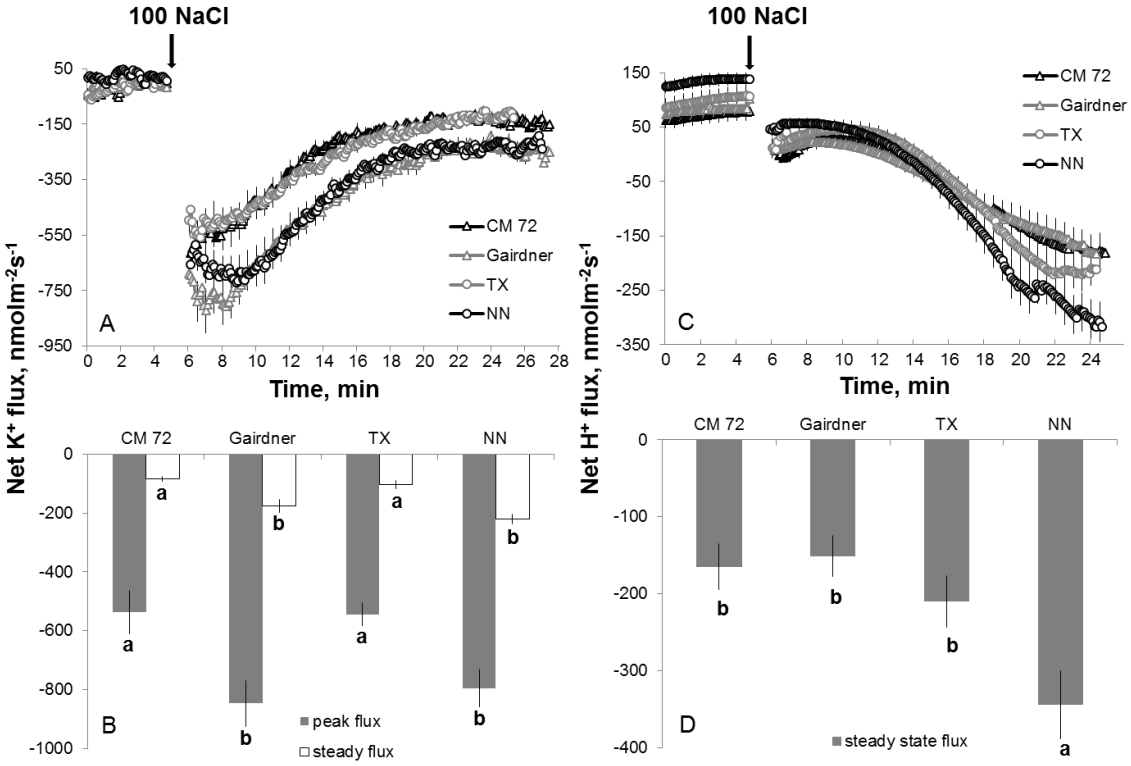


Fig. 6. Net K^+ (A, B) and H^+ (C, D) flux responses measured from the mesophyll tissue of 4 barley cultivars contrasting in salinity tolerance (CM72 and TX9425 – tolerant; NN and Gairdner – sensitive). To expose mesophyll, the excised leaf (middle part) was cut to segments (roughly 5 × 8 mm) by a sharp blade angularly. (A) kinetics of K^+ flux responses; (B) steady-state (20 min after stress onset) and peak K^+ flux values; (C) kinetics of H^+ flux responses; (D) steady-state H^+ flux values. Mean ± SE (n = 14-46). Within each treatment different low case letters indicate significant differences by Duncan's multiple range test at $p < 0.05$.

3.3.3 The observed effects are related to specific ionic but not osmotic component of salt stress

Acute 100mM NaCl treatment causes rapid cell shrinking thus concentrating the internal K^+ and other solutes and creating a chemical gradient favoring passive K^+ leak from the cell.

To ensure that the observed difference in K^+ retention is not attributed to genetic variability in elasticity modules among genotypes but is indeed related to regulation of plasma membrane K^+ transporters, additional experiments were undertaken using 170mM mannitol (a non-ionic osmolyte isotonic to 100mM NaCl). Contrary to NaCl treatment, all plants took up but not lost K^+ in response to isotonic mannitol treatment (Fig. 7A), suggesting that rapid changes in cell volume were not the reasons behind observed the NaCl-induced K^+ efflux. These results are consistent with our previous observation on both root (Shabala and Lew, 2002; Chen et al., 2005) and mesophyll (Shabala, 2000) tissues showing high specificity of observed ion flux responses. Moreover, no significant (at P <0.05) genotypic difference in osmotic adjustment was found between cultivars, with all genotypes increasing their net K^+ uptake by approximately 200 nmolm^{-2} s^{-1} (Fig. 7B). Thus, the observed effects are indeed related to specific ionic but not osmotic component of salt stress.

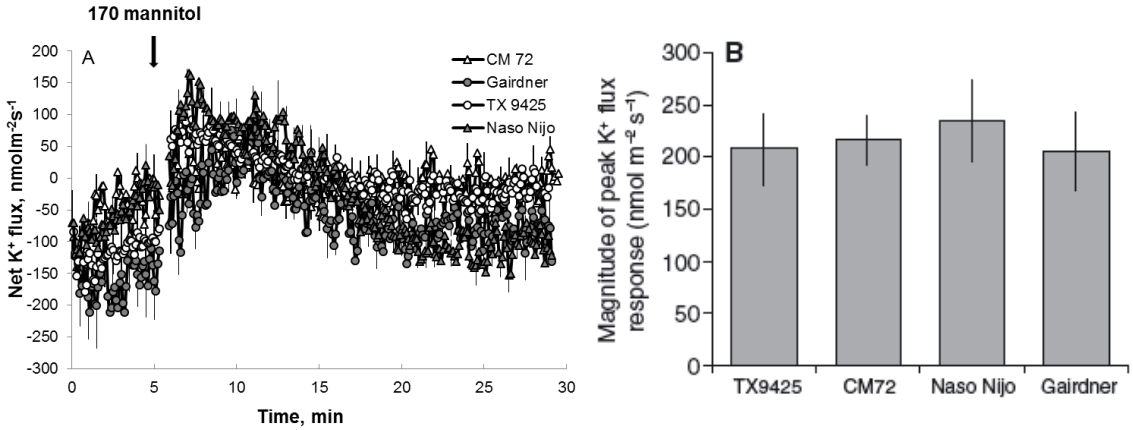


Fig. 7. Net K^+ fluxes measured from the mesophyll tissue of four barley cultivars contrasting in salinity tolerance (CM72 and TX9425, tolerant; NN and Gairdner, sensitive) in response to 170m*M* mannitol treatment (isotonic to 100m*M* NaCl). (A) Kinetics of K^+ flux responses; (B) the magnitude of K^+ flux response; [a difference between steady-state (before stress) and peak K^+ flux values]. Mean ± SE (n = 6).

3.3.4 Pharmacology data suggests involvement of voltage-gated K^+ permeable channels in K^+ retention in leaves

The magnitude of the peak NaCl-induced K^+ flux from leaf mesophyll was strongly (~ 70%) inhibited by TEA, a known blocker of K^+-permeable plasma membrane channels (Shabala et al., 2007b) (Fig. 8A, B). Leaf pre-treatment with 1 mM vanadate (a known blocker of P-type H^+-ATPase), exacerbated detrimental effects of salinity on K^+ retention in leaf mesophyll. Both peak- and steady-state K^+ efflux values were significantly higher (Fig. 8A, B), suggesting that membrane potential maintenance provided by H^+-ATPase plays an important role in controlling K^+ fluxes across the mesophyll plasma membrane. Consistent with this notion, vanadate treatment significantly reduced the magnitude of NaCl-induced H^+ efflux from mesophyll tissue (Fig. 8C, D). Interestingly, however, leaf pre-treatment with TEA also reduced the magnitude of NaCl-induced H^+ efflux, suggesting a possibility of a feedback control over H^+-ATPase activity by cytosolic K^+.

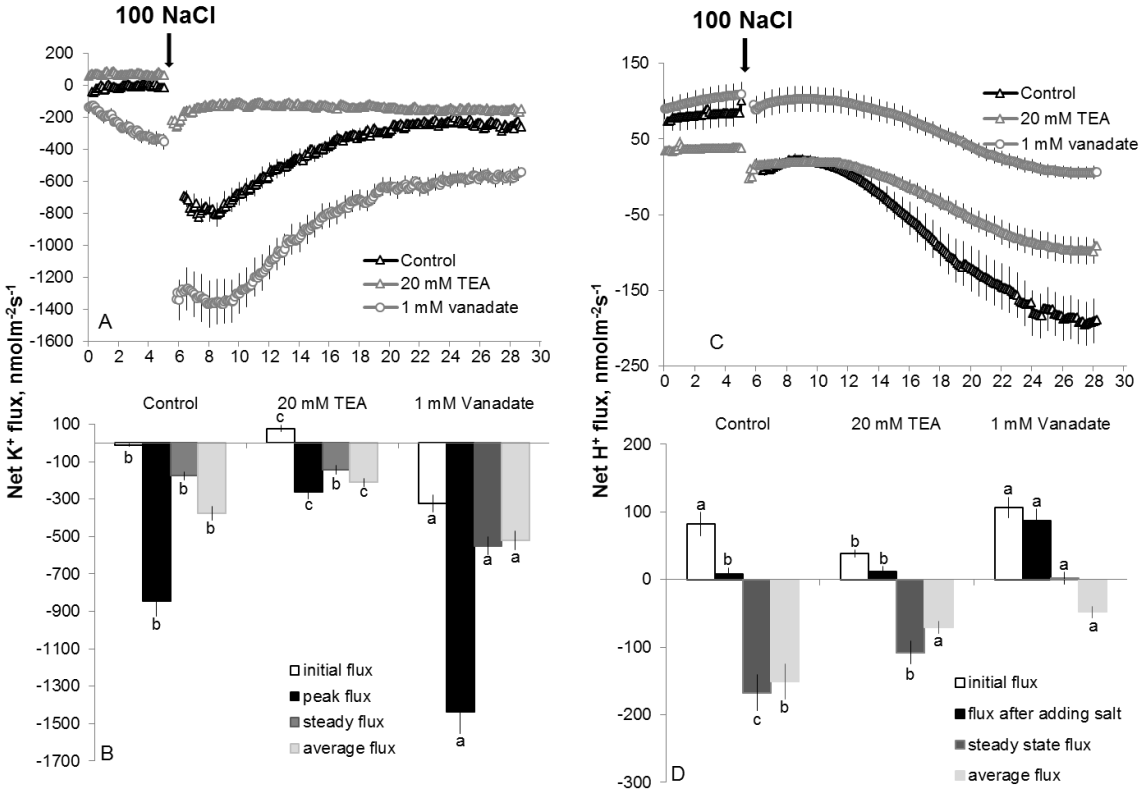


Fig. 8. Ion fluxes measured from mesophyll after cutting barley leaf (Gairdner). (A) transient net K^+ fluxes; (B) calculation of K^+ fluxes; (C) transient net H^+ fluxes; and (D) calculation of H^+ fluxes. Means ± SE (n=24-30). Within each treatment different low case letters indicate significant differences by Duncan's multiple range test at $p < 0.05$.

3.4 Discussion

3.4.1 Potassium retention in mesophyll is essential for salinity tolerance

The previous work in our laboratory has emphasised the importance of K^+ retention in roots of a range of plant species, including barley (Chen et al., 2005; Chen et al., 2007c, d) and wheat (Cuin et al., 2008; Cuin et al., 2012). Here we report that K^+ retention in leaf mesophyll is also essential to confer salinity stress tolerance in these species (Fig. 5, 6). At the cellular level, K^+ catalyses many metabolic reactions and there are at least 80 enzymes known that require K^+ (Evans and Sorger, 1966; Suelter, 1970); most of these are present in leaf mesophyll. Thus, inability of mesophyll cells to retain K^+ will result in substantial decline in leaf photochemistry, ultimately affecting plant growth and biomass accumulation. In addition to activation of enzymes, K^+ is used as a major counter ion for cytoplasmic polyanions, control of photoassimilate loading into phloem, and is essential for the formation and maintenance of plasma membrane potential and electrogenic process in chloroplast membranes (Hedrich and Schroeder, 1989; Fang et al., 1995; Marschner et al., 1996; Talbott and Zeiger, 1996). Potassium also plays a critical role in determining cell fate and transition to PCD, by controlling caspase (in animal systems; Hughes and Cidlowski, 1999) or protease and endonuclease (in plant systems; Demidchik et al., 2010) activity. It was shown earlier that Arabidopsis *gork* mutants lacking outward rectifying depolarization-activated K^+ (KOR) channels and, thus capable to retain more K^+ under saline conditions, had less than 1/3 of TUNEL-stained nuclei compared with wild type plants (Demidchik et al., 2010). We believe that a similar scenario is applicable in the case of leaf mesophyll in wheat and barley, and that salt-induced damage to leaf lamina observed as necrotic spots on leaf tips in wheat and barley may be a result of mesophyll cells undergoing PCD as a result of K^+ loss. Direct quantification of PCD related events (such as cytochrome C release; nuclei TUNEL staining, or DNA laddering) is needed to support this hypothesis.

3.4.2 Downstream targets

Several types of K^+ permeable channels at the mesophyll plasma membrane may mediate K^+ efflux upon salinity stress (Shabala, 2003). This includes Shaker-like outward-rectifying K^+-selective KOR channels and non-selective cation channels (NSCC). KOR channels (GORK in Arabidopsis) are activated by membrane depolarisation at potentials more positive than E_k (Hosy et al., 2003; Véry and Sentenac, 2003). NSCCs appear to form a large, heterogeneous group of channels, with 40 putative NSCCs revealed in the Arabidopsis genome sequence (Mäser et al., 2001; Demidchik et al., 2002). Most of these are only weakly voltage-dependent but may be activated by other

factors such as mechanical tension, calcium, cyclic nucleotides and glutamate (Demidchik et al., 2002; Demidchik and Maathuis, 2007). NSCCs discriminate poorly between K^+ and Na^+ (K^+/Na^+ selectivity ratios between 0.3 and 3, Demidchik et al., 2002) and, thus, could mediate both Na^+ uptake and K^+ efflux under saline conditions.

It was shown that acute NaCl treatment results in substantial depolarisation of plasma membrane in leaf mesophyll (e.g. beans – Shabala, 2000; Arabidopsis - Shabala et al., 2006; tobacco – Shabala et al., 2007b). Thus, accumulation of substantial amounts of NaCl in the leaf apoplast may activate KOR channels and result in K^+ leakage from the mesophyll. Our pharmacological data reported here suggests that this scenario is also true for barley mesophyll. Indeed, K^+ leakage was substantially suppressed by using TEA, a known blocker of KOR channels (Fig. 8) but enhanced in leaves pre-treated with orthovanadate, a known inhibitor of P_2B type of H^+-ATPase. H^+-ATPase is primarily responsible for maintenance of plasma membrane potential (Sze et al., 1999), and suppression of its activity prevents plant ability to restore (otherwise depolarised) membrane potential, resulting in larger K^+ leakage via depolarization-activated KOR channels.

The overall amount of K^+ leaked from wheat mesophyll was substantially smaller compared with appropriate amount leaked from barley (see Figs 5and 6 for comparison). This is somewhat counterintuitive, given the fact that barley is always classified as more tolerant to salinity compared with wheat (Greenway and Munns, 1980). However, these findings are in good agreement with our early observations on wheat and barley roots. Indeed, the magnitude of NaCl-induced K^+ loss from wheat roots was only approx. 10% of that measured in barley under similar conditions (Cuin et al., 2008). Moreover, while K^+ retention in barley roots showed a strong positive correlation with activity of the plasma membrane H^+-ATPase and its ability to maintain more negative membrane potential (Chen et al., 2007c), no such correlation was found in wheat (Cuin et al., 2008). It may be suggested, therefore, that the pathway of K^+ leakage from both root and leaf tissues differ substantially between wheat and barley. In barley, voltage-gated KOR channels are obviously the dominant mechanisms (Chen et al., 2007c) and these are activated immediately upon acute NaCl treatment as discussed above. In wheat, the major bulk of K^+ leakage may occur through NSCC. These channels show only weak voltage dependence (Demidchik and Maathuis, 2007) and as such, are not expected to be dramatically activated by Na^+ entering the cell. However, NSCC channels are known to be directly activated by various forms of ROS (Demidchik et al., 2003, 2007), and ROS

accumulation in both plant root and leaf tissues (Cavalcanti et al., 2007) is widely reported under saline conditions. Moreover, ROS accumulation is a much slower process compared with the almost instantaneous membrane depolarization upon acute saline stress. As such, ROS-induced activation of NSCC will be more gradual and take much longer then instantaneous activation of voltage-gated KOR channels. This may explain larger K^+ leakage from barley compared with wheat tissues under conditions of acute salt stress in our experiments. Under long-term stress conditions, however, ROS-induced K^+ leak may become more essential, which may explain generally higher salt sensitivity of wheat compared with barley (Munns and Tester, 2008).

3.4.3 Suitability for screening

Our finding of importance of K^+ retention in leaf mesophyll for salinity tolerance in cereals may be of significant importance to plant breeders. To be useful in MAS-based breeding programs, however, this trait should be reliably estimated using some relatively inexpensive and user-friendly high-throughput technology. The non-invasive MIFE technique show great potential of development as such high-throughput tool. In principle, simple measurements of K^+ concentration in the external solution after adding NaCl to stress the plants by a commercial ion-selective electrode may potentially be used as a cheap proxy for this method. However, the resolution of this method will be at least an order of magnitude lower. The method is also prone to significant methodological errors as the biggest changes detected are in the vicinity of mesophyll tissue, and positioning the macroelectrode at a fixed and close distance to the tissue may be technically challenging. Thus, while MIFE technique-based measure of NaCl-induced K^+ efflux from leaf mesophyll was recommended remains the most preferred current method, the feasibility of using simple K^+ concentration measurements are worth a proper investigation.

In this work, ion flux responses were measured from both epidermal and mesophyll tissues, with consistent results. For obvious reasons, measuring NaCl- induced K^+ efflux in mesophyll is easier to conduct, as these measurements eliminate a requirement for additional labour- and time-consuming procedures of removing epicuticular waxes. Cutting a leaf segment and exposing mesophyll tissue takes only a few seconds; immobilising these segments in prefabricated Perspex holders is also a matter of seconds and requires no special skills.

Net K^+ fluxes were measured in this study for up to 30 min upon NaCl addition. However, for screening purposes, much shorter (e.g. 2-3 minutes) measurements will

suffice. Indeed, peak K^+ flux responses occurred at ~ 1.5 min after NaCl treatment (Fig. 6B), and peak values were proven to be a sensitive physiological index for tissue tolerance in barley (Fig. 6). Thus, even allowing several minutes for electrode positioning and manipulation in the chamber, one complete record will require no longer then 6-7 min (including immobilization time). The current MIFE configuration allows parallel recording of up to 3 specimens; this amounts into 25 to 30 specimens per hour, or 160 to 200 specimens per standard business day. Moreover, existing MIFE electronics has an 8-channel amplifier, and the above limitation of 3 channels is caused merely by the type of manipulator used for electrode positioning. For a relatively modest cost, the latter can be automated (e.g. using robotic arm) enabling 8 specimens to be processed simultaneously. Altogether, it will increase the screening capacity to over 500 specimens per day, which is large enough to be termed as high-throughput.

Chapter 4

K^+ retention in leaf mesophyll, an overlooked component of salinity tolerance mechanism: a case study for barley[#]

Abstract

Plant salinity tolerance is a physiologically complex trait, with numerous mechanisms contributing to it. In this work we show that the ability of leaf mesophyll to retain K^+ represents an important and essentially overlooked component of a salinity tolerance mechanism. The strong positive correlation between mesophyll K^+ retention ability under saline conditions (quantified by the magnitude of NaCl-induced K^+ efflux from mesophyll) and the overall salinity tolerance (relative fresh weight and/or survival or damage under salinity stress) was found while screening 46 barley genotypes contrasting in their salinity tolerance. Genotypes with intrinsically higher leaf K^+ content under control conditions were found to possess better K^+ retention ability under salinity and, hence, overall higher tolerance. Contrary to previous reports for barley roots, K^+ retention in mesophyll was not associated with an increased H^+-pumping in tolerant varieties but instead correlated negatively with this trait. These findings are explained by the fact that increased H^+ extrusion may be needed to charge balance the activity and provide the driving force for the high affinity HAK/KUP K^+ transporters required to restore cytosolic K^+ homeostasis in salt-sensitive genotypes.

Keywords

Cytosolic K^+ homeostasis; ion flux; membrane potential; membrane transport; tissue tolerance

4.1 Introduction

K^+ is recognized as a rate-limiting factor for crop yield and is the major cationic inorganic nutrient in non-halophytes (Maathuis and Amtmann, 1999; Shabala, 2003; Pettigrew, 2008; Dreyer and Uozumi, 2011), comprising generally 4 - 6% of plant dry matter. K^+ plays an important role in plants response to major abiotic stresses such as drought, salinity, cold and frost, and waterlogging (Cakmak, 2005; Wang et al., 2013; Shabala and Pottosin, 2014).

[#], This chapter has been published as: Wu H, Zhu M, Shabala L, Zhou M, Shabala S (2015) Journal of Integrative Plant Biology 57: 171–185.

In broad terms, plants salinity stress can be divided into two phases: osmotic phase (rapid response to change in osmotic potential) and ionic phase (gradual Na^+ accumulation) (Fortmeier and Schubert, 1995; Munns et al., 2002; Munns and Tester, 2008). Various mechanisms are employed by glycophytes to deal with detrimental effects of salinity including Na^+ extrusion from uptake, Na^+ sequestration in vacuoles, osmotic adjustment, control of xylem loading of Na^+, its retrieval from the shoot, and reactive oxygen species (ROS) detoxification (reviewed in Tester and Davenport, 2003; Colmer et al., 2005; Munns et al., 2006; Munns and Tester, 2008; Shabala and Pottosin, 2014). Accordingly, numerous attempts have been undertaken to modify plant salinity tolerance by tampering with one of the above mechanisms, both by transgenic (e.g. increasing *de novo* synthesis of organic osmolytes, Huang et al., 2012 ; overexpressing tonoplast NHX Na^+/H^+ exchangers to improve vacuolar Na^+ sequestration, Apse et al., 1999; enhanced Na^+ extrusion from roots by over-expressing SOS1 plasma membrane Na^+/H^+ antiporter, Shi et al., 2003) and traditional (quantitative trait loci (QTL) mapping for Na^+ exclusion from the shoot; Lindsay et al., 2004; Genc et al., 2010; Munns et al., 2012). However, the progress is still much less than one would hope.

Another important trait that emerged over the last decade is K^+ retention in roots (Shabala and Cuin, 2008). Salt tolerant barley variety showed higher K^+ content in root than salt sensitive variety when grown under saline conditions (Liang, 1999). A strong positive correlation between root's ability to retain K^+ when challenged with NaCl and overall plant salinity tolerance was reported for several species including barley (Chen et al., 2005, 2007b, c), wheat (Cuin et al., 2008, 2012), lucerne (Smethurst et al., 2008), and poplar (Sun et al., 2009). The essentiality of the above correlation was attributed to the critical role of cytosolic K^+ homeostasis for a broad range of metabolic processes in root cells (Shabala and Cuin, 2008).

Leaf mesophyll is central to photosynthetic plant performance and, ultimately, biomass gain. Plant performance under stress conditions largely depends on efficiency of photosynthetic machinery. In this context, cytosolic K^+ homeostasis in mesophyll cell may be essential to maintenance of optimal photosynthetic performance (Carkmak, 2005). Reduced CO_2 assimilation capacity was reported for K^+ deficient cotton (Bednarz and Oosterhuis, 1999), hickory (Jin et al., 2011), sugar beet (Terry and Ulrich, 1973), and barley (Degl'Innocenti et al., 2009) species. Also, it was estimated that an expanding leaf cell has to accumulate solutes at a rate of~ 66 mosmol kg^{-1} h^{-1}; 50% of this quantity is attributed to accumulation of K^+ (Boscari et al., 2009). Thus, the accumulation rates of K^+

in leaf cells may limit the rate of its expansion (Volkov et al., 2009), especially under saline conditions. The importance of maintaining high shoot K/Na ratio was repeatedly suggested as a hallmark feature of salt-tolerant crops (Dasgan et al., 2002; Ren et al., 2005; Hauser and Horie, 2010). Ball et al. (1987) showed that salinity induced K^+ deficiency caused a loss of functional photosystem II in leaves of grey mangrove. Similar result was found in spinach, where the photosynthetic capacity was related to the bulk lamina K^+ content (Chow et al., 1990).

While most salinity-related papers quantified shoot K/Na ratio on a whole-tissue basis, the importance of intracellular and intercellular ion sequestration should be not overlooked (Leigh and Storey, 1993; Fricke et al., 1994, 1996). K^+ is actively transported into the vacuole of both epidermal and mesophyll cells (Cuin et al., 2003; Bassil et al., 2011) and can be potentially used as a buffer to compensate for salt-induce K^+ reduction in the cytosol. Vacuolar K^+ concentrations in barley leaf mesophyll decreased by 50-80 mM during NaCl treatment (Fricke et al., 1996; Cuin et al., 2003). At the same time, cytosolic K^+ has been barely changed by salinity (a decline in a(K) from 79 to 64 mM; Cuin et al., 2003). However, it appears that not all vacuolar K^+ is available to be used for cytosolic K^+ homeostasis maintenance in leaf mesophyll (Fricke et al., 1996), making a need to draw K^+ from extracellular sources. One of these sources is leaf epidermis.

In barley, leaf epidermal cells occupy about 27% of the total leaf symplastic volume (Dietz et al., 1992), and the patterns of ion distribution differ dramatically between mesophyll and epidermis in barley leaves (Huang and van Steveninck, 1989; Dietz et al., 1992; Fricke et al., 1996; Leigh and Storey, 1993; Leigh and Tomos, 1993; Fricke et al., 1996; Karley et al., 2000; Conn and Gilliham, 2010). Contrary to mesophyll, epidermal cells do not require a minimum vacuolar K^+ concentration of 20-60 mM to keep the cytosolic K^+ concentration at a metabolically sustainable level (Fricke et al., 1996), and a 5-fold decrease in both vacuolar and cytosolic K^+ concentrations was reported for epidermal leaf cells grown under saline conditions (Cuin et al., 2003). Using an X-ray microanalysis technique, Leigh and Storey (1993) showed that at low shoot K^+ concentrations, K^+ was preferentially located in mesophyll cells, with relatively few epidermal cells having detectable levels of this element. Given the fact that salinity can reduce shoot tissue K^+ content by several fold (Muralitharan et al., 1992), the importance of maintaining cytosolic K^+ homeostasis under saline conditions is beyond any doubt.

Boscari et al (2009) have identified three major K^+ transport systems operating in barley leaf mesophyll. HvAKT2 inward-rectifying K^+ channels showed strongest

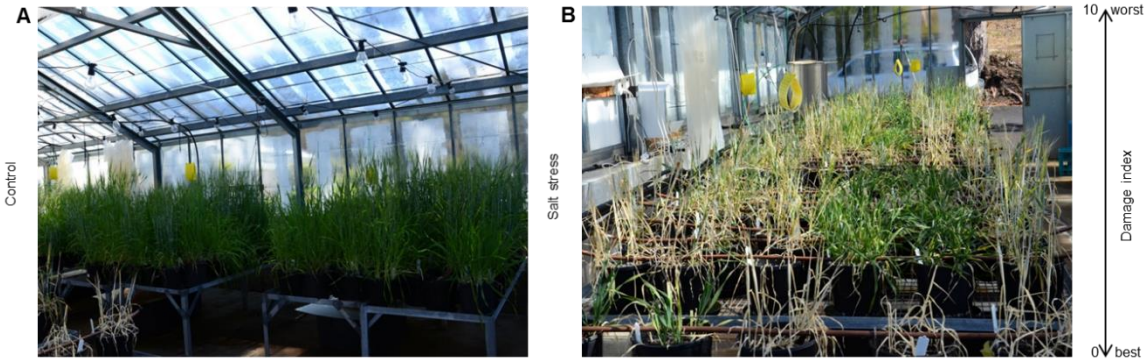
expression in leaf tissue, followed by the high-affinity HvHAK4 K^+ transporter. HvAKT1 expression in leaf was weak, only 3-4 % as compared with roots (Boscari et al., 2009). Interestingly, however, no significant changes in gene expression levels were found in leaf mesophyll tissue (Boscari et al., 2009), despite major changes in K^+ concentrations and osmolality of cells. These results are consistent with our observation of roots (Shabala and Cuin, 2008) and suggest that it not increased K^+ uptake but instead K^+ retention in mesophyll cell that matters.

Despite a clear evidence for leaf mesophyll cells possessing highly efficient mechanisms for maintaining cytosolic K^+ homeostasis (see above), to the best of our knowledge no attempt to evaluate and validate K^+ retention ability of leaf mesophyll as a trait contributing to overall salinity stress tolerance was conducted for any species at a scale sufficient to convince breeders. As the very best, all the previous papers reported a link between the overall shoot potassium content and salinity tolerance in barley (Liang, 1999; Chen et al., 2005, 2007d; Flowers and Hajibagheri, 2001) but stop short of characterising the extent of net K^+ exodus from the cytosol into apoplastic space. With many confounding factors involved (e.g. genotypic- and stress-related difference in root K^+ acquisition; radial transport; xylem loading; and delivery to the shoot), establishing the role of K^+ retention in mesophyll as a component of tissue tolerance mechanisms was not possible by the whole-organ level.

In this work, we have extended our seminal work using just a few contrasting barley varieties (Wu et al., 2013) to undertake a large-scale validation of the essentiality of K^+ retention in leaf mesophyll as a component of salinity tolerance mechanism in barley. By screening nearly 50 barley genotypes contrasting in their salinity tolerance, we are reporting the strong positive ($r > 0.55$, $P < 0.01$) correlation between mesophyll K^+ retention ability under saline conditions (quantified by the magnitude of NaCl-induced K^+ efflux from mesophyll) and the overall salinity tolerance (relative fresh weight and/or survival or damage under salinity stress). We also show that, contrary to previous reports for barley roots (Chen et al., 2007c), K^+ retention in mesophyll was not related to increased H^+-pumping in tolerant varieties. These findings are explained by the fact that increased H^+ extrusion may be needed to charge balance the activity and provide the driving force for the high affinity HAK/KUP K^+ transporters required to restore cytosolic K^+ homeostasis in salt-sensitive genotypes.

4.2 Results

4.2.1 Large variability in growth performance under saline conditions



Suppl. Fig. S1. Genetic variability of salinity tolerance in barley after 40 days salt treatment (300 mM NaCl). (A) Control (plants irrigated with tap water); (B) Salinity treatment (plants irrigated with 300 mM NaCl).

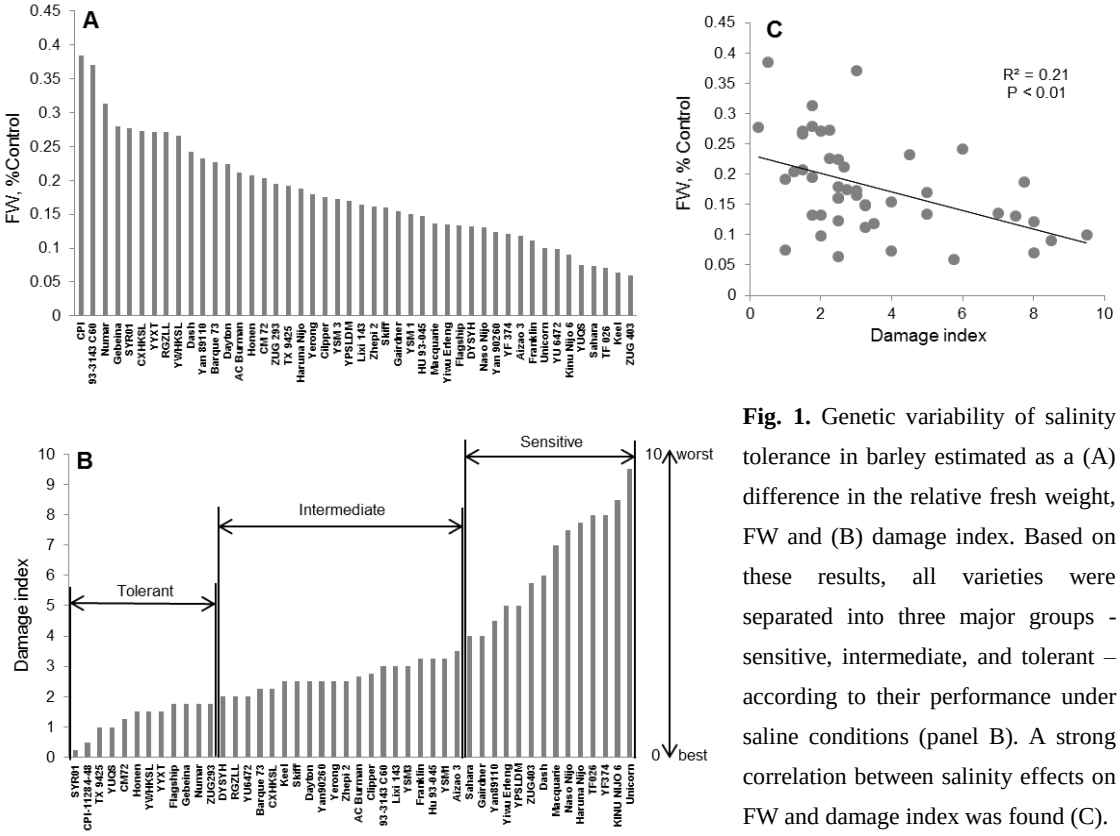


Fig. 1. Genetic variability of salinity tolerance in barley estimated as a (A) difference in the relative fresh weight, FW and (B) damage index. Based on these results, all varieties were separated into three major groups - sensitive, intermediate, and tolerant – according to their performance under saline conditions (panel B). A strong correlation between salinity effects on FW and damage index was found (C).

A dramatic difference in plant growth was observed between barley varieties grown under 300 mM NaCl treatment for 40 days in a glasshouse (Suppl. Fig. S1). This was further confirmed by measuring agronomical characteristics such as fresh weight (FW) and plant damage index (Fig. 1). A six-fold variation was found between relative FW values of the most and the least tolerant barley varieties, with values ranging from 0.06 (e.g. 94% reduction compared with control) to 0.38 (e.g. 62% reduction) (Fig. 1A). Large variability was also observed in the damage index of barley varieties tested, with the damage index ranging from 0.25 (most tolerant) to 9.5 (least tolerant) (Fig. 1B). Based on

the latter results, all barley varieties were grouped into three major clusters: (i) sensitive (damage index 4 to 10), (ii) intermediate (damage index 2 to 4), (iii) tolerant (damage index < 2). Wild-type barleys were clustered as a sub-class of the tolerant group (index 0-0.6) (Fig. 1B). Significant negative correlation (r = 0.46, $P < 0.01$) was found between relative FW and damage index (Fig. 1C), suggesting that either of these indices may be used as an indicator of salinity tolerance.

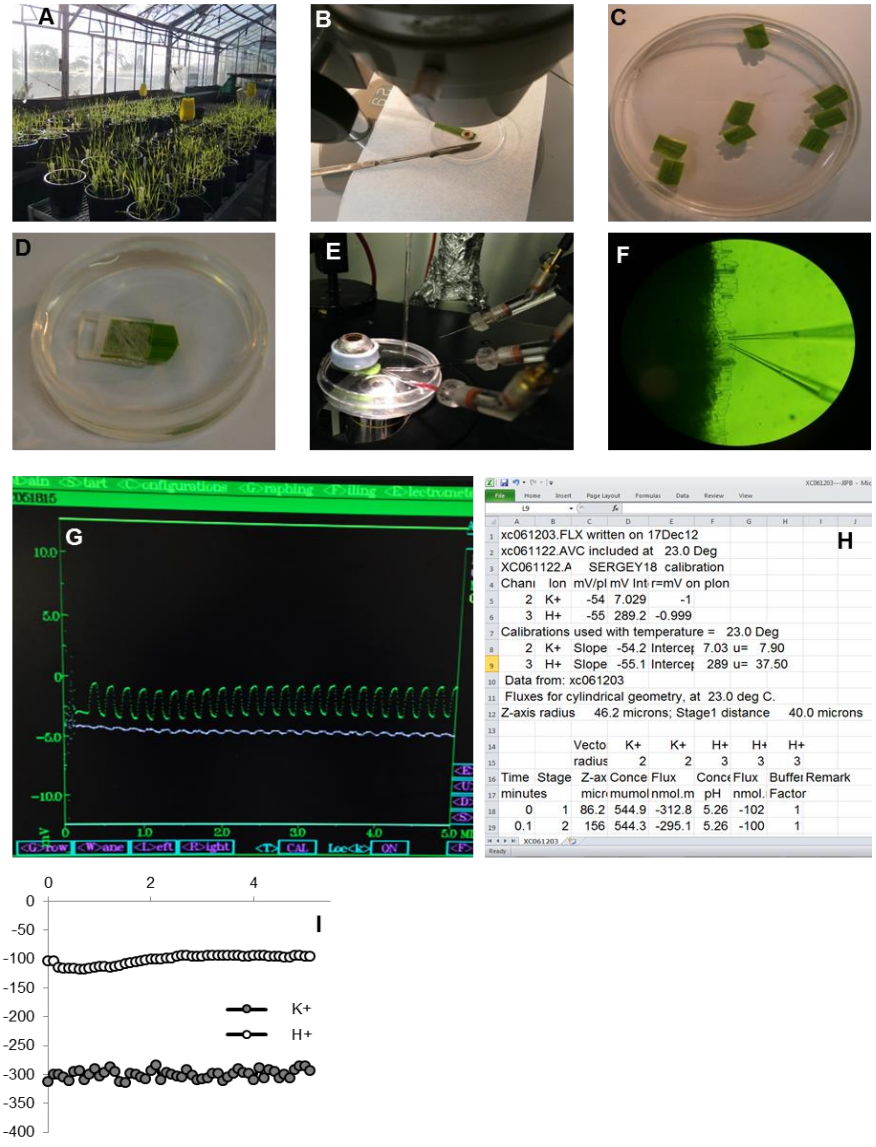


Fig. 2. Screening barley plants for K^+ retention in leaf mesophyll. Nine steps of experimental procedure are illustrated. (A) Plants are grown in the glasshouse; (B) cross–sectional cut is made on the excised leaf; (C) prepared cut samples are floated on the surface of BSM solution and kept in the dark overnight; (D) samples are mounted in a Perspex holder and treated with 100 mM NaCl for 0.5 h; (E) samples are transferred to the microscope stage into the Faraday cage; (F) ion selective electrodes are positioned next to exposed mesophyll; (G) net K^+ and H^+ fluxes are measured and displayed on the screen; (H) measured electrical signals are converted in net fluxes (in nmol m^{-2} s^{-1}) and tabulated in an Excel-compatible format; (I) appropriate graphs are plotted.

4.2.2 NaCl-induced K^+ flux from leaf mesophyll correlates with salinity tolerance in barley

The protocol of measuring NaCl-induced K^+ flux from leaf mesophyll via MIFE technique is illustrated in Fig. 2. 30 min exposure of leaf mesophyll to 100 mM NaCl had resulted in a massive K^+ leak from the tissue, ranging from - 94 (in variety YWHKSL) to - 696 nmol m^{-2} s^{-1} (in Aizao 3) (Fig. 3A). Significant positive correlation (r = 0.37, P < 0.05) was found between leaf K^+ retention ability (lower K^+ efflux) and relative FW (Fig. 3B). Also significant was a correlation between leaf K^+ retention ability and the damage index (Fig. 3C), with better performing varieties (lower damage index) showing less K^+ efflux. Interestingly, the extent of this correlation is dependent on the cluster the plant belonged to. All tolerant and some intermediate varieties were more "robust" in terms of their K^+ flux responses to NaCl (e.g. relatively small variability in the damage index, despite huge variation in NaCl-induced K^+ flux). These varieties are termed as Group 1 in Fig. 3C. All sensitive (and some intermediate) varieties (termed as Group 2 in Fig. 3C) showed, on the contrary, much stronger dependence of their overall the damage index scoring from their ability to retain K^+ in leaf mesophyll under saline exposures. In quantitative terms, both groups had high and significant (P < 0.01) correlation between K^+ efflux and the damage index, but the slopes between two groups differed dramatically (-0.0167 in Group 2 vs -0.0039 in Group 1). The physiological rationale behind this observation may be that tolerance varieties may possess some other (additional) mechanisms to deal with salinity, while sensitive varieties rely heavily on K^+ retention (e.g. tissue tolerance) for their survival.

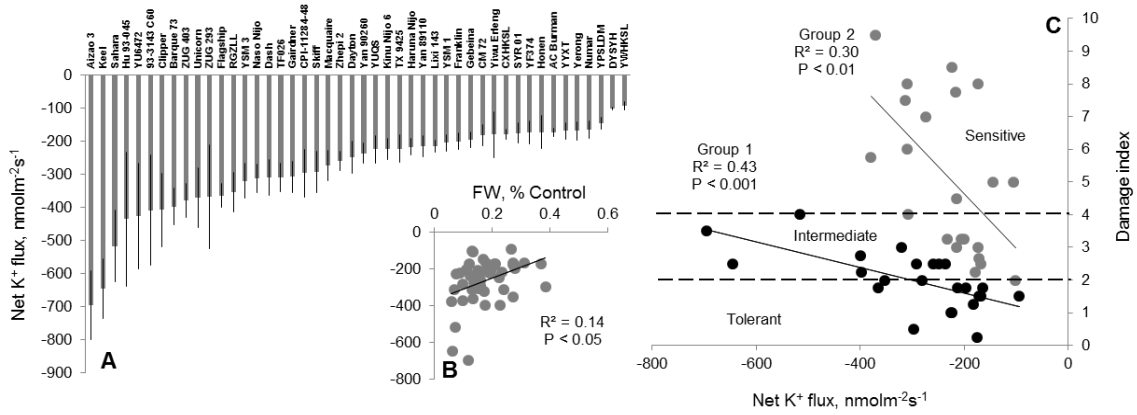


Fig. 3. Genetic variability in the ability of barley leaf mesophyll to retain K^+ under saline conditions. (A) - steady-state net K^+ fluxes measured from mesophyll cells after 30 min of 100 mM NaCl exposure. Mean ± SE (n=6−8). (B) - correlation between net K^+ flux from leaf mesophyll and relative FW. (C) - correlation between net K^+ flux and damage index. Here, all varieties were clustered into two groups based on their differential ability to retain K^+ upon salinity exposure. Each point represents a separate variety.

4.2.3 K^+ content correlates with K^+ retention in mesophyll and salinity tolerance

To better understand importance of the leaf K^+ retention in plant salinity tolerance, we further measured K^+ content in all barley varieties grown under control conditions. A two-fold difference was found in the leaf K^+ content ranging from 0.84 to 1.61 mmol g^{-1} DW (Fig. 4A). When we compared leaf K^+ content and the damage index, a weak but significant positive correlation (r = 0.33, $P < 0.05$) between leaf K^+ content and relative FW was found (Fig. 4B). Taken together, these results suggest that, in addition to its ability to prevent K^+ loss from mesophyll, the initial K^+ status is also an important determinant of salinity tolerance mechanism. Consistent with this notion, a significant positive correlation (r = 0.33, $P < 0.01$) was found between leaf K^+ content and NaCl-induced K^+ efflux (Fig. 4C), suggesting a possible causal relationship between leaf K^+ content and plants ability to prevent NaCl-induced K^+ loss in mesophyll.

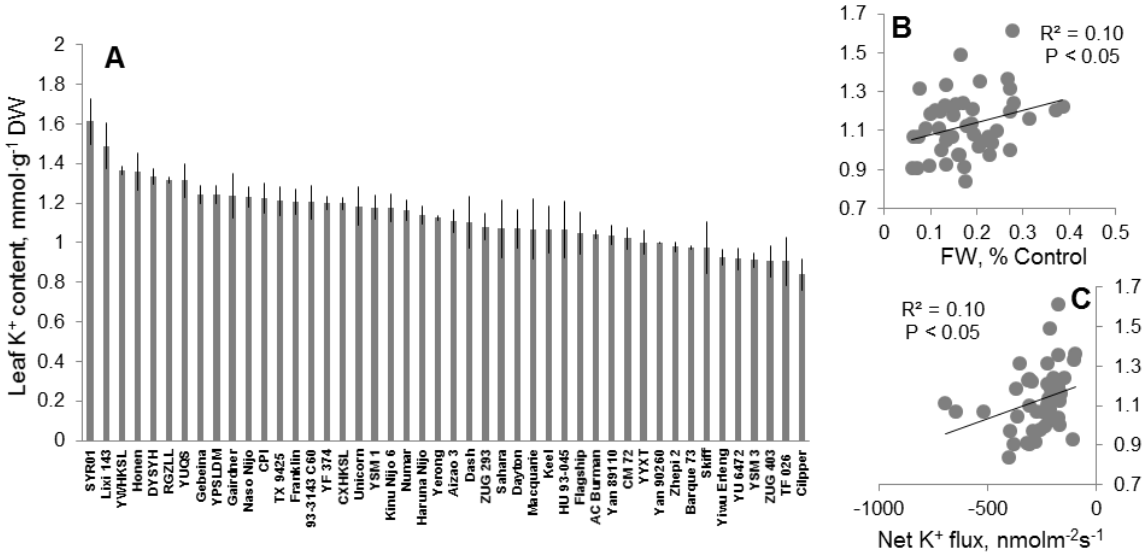


Fig. 4. Genetic variability in leaf K^+ content in barley plants grown under control condition and its relationship with the overall salinity tolerance. (A) - leaf K^+ content (mmol·g^{-1} DW). Mean ± SE (n=3). (B) - correlation between leaf K^+ content and relative FW of plants grown under saline conditions (300 mM for 40 days). (C) - correlation between leaf K^+ content and K^+ retention in leaf mesophyll (estimated as net K^+ efflux upon 100 mM NaCl treatment). Each point represents a separate variety.

4.2.4 Reduced H^+ efflux and increased leaf K^+ retention correlate with FW and survival in barley

To gain insights into mechanisms underlying differential NaCl-induced K^+ efflux from barley mesophyll, NaCl-induced H^+ fluxes were also investigated. A broad variability of NaCl-induced H^+ flux was found, with steady-state values ranging from +38 (net uptake; in ZUG293 variety) to -587 nmol m^{-2} s^{-1} (net efflux; in Aizao 3) (Fig. 5A). Contrary to

previous observations on roots (Chen et al., 2007b), highly significant negative correlation was found between H^+ flux and relative FW (r = 0.42, $P < 0.01$; Fig. 5B). Net K^+ efflux correlated positively with net H^+ efflux (r = 0.39, $P < 0.01$; Fig. 5C). It has previously been showed that NaCl-induced H^+ flux from barley leaf mesophyll was significantly inhibited by vanadate pre-treatment (Wu et al., 2013), implicating the involvement of H^+-ATPase activity in this process.

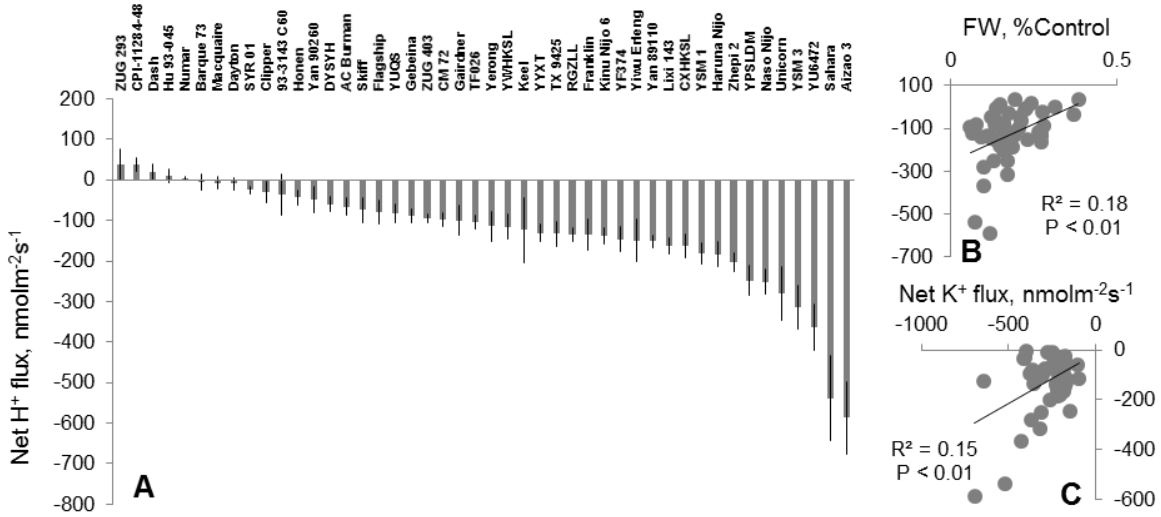


Fig. 5. Genetic variability in the magnitude of NaCl-induced H^+ flux responses in barley. (A) - steady-state net H^+ fluxes measured from mesophyll cells after 30 min of 100 mM NaCl exposure. Mean ± SE (n=6−8). (B) - correlation between net H^+ flux and relative FW of plants grown under saline conditions (300 mM for 40 days). (C) - correlation between net H^+ and K^+ fluxes measured from mesophyll cells after 30 min of 100 mM NaCl exposure. Each point represents a separate variety.

4.2.5 NaCl-induced ion flux patterns correlate with the ability of barley to maintain shoot water content

Similar to other physiological parameters measured, high variability was found in shoot water content (SWC) among salt treated barley varieties screened, ranging from 0.52 (in variety ZUG 403) to 0.81 (in SYR 01) (Fig. 6A). Highly significant negative correlation was found between SWC and the damage index (r = 0.46, $P < 0.01$; Fig. 6B). A significant positive correlation was found between leaf K^+ content and SWC (r = 0.39, $P < 0.01$; Fig. 6C), and between mesophyll K^+ retention and SWC (r = 0.33, $P < 0.05$; Fig. 6D), suggesting that ability of a plant to maintain K^+ level in the leaf mesophyll may be instrumental in determining shoot water status under salinity stress. It also echoes the possible causal relationship between leaf K^+ content and plants ability to prevent NaCl-induced K^+ loss in mesophyll. Further statistical analyses showed significant negative correlation between H^+ flux and SWC (r = 0.47, $P < 0.01$; Fig. 6E).

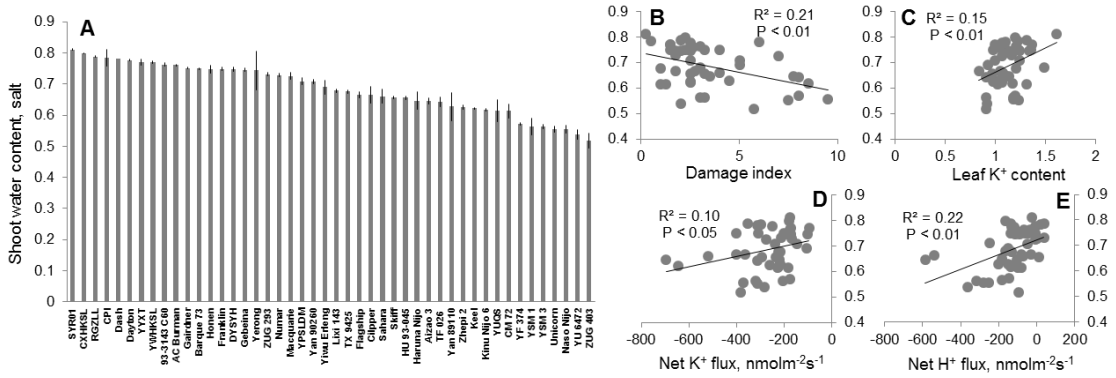


Fig. 6. Genetic variability in the shoot water content (SWC; % control) in barley and its relationship with overall salinity tolerance. (A) - all genotypes are ranked according to their SWC. (B) - correlation between SWC and damage index. (C) - correlation between SWC and K^+ retention ability of leaf mesophyll estimated as net K^+ efflux upon 100 mM NaCl treatment. (D) - correlation between SWC and NaCl-induced steady state H^+ flux. Each point represents a separate variety.

4.3 Discussion

4.3.1 K^+ retention in leaf mesophyll constitutes an important component of salinity tolerance mechanism

K^+ plays an important role in plant life acting as a dominant counterion to balance the negatively charged proteins and nucleic acids as well as being involved in enzyme activation, protein synthesis stabilization, membrane potential formation, maintenance of turgor pressure and cytosolic pH homeostasis, and mediating all types of plant movements (Shabala, 2003; Véry and Sentenac, 2003; Dreyer and Uozumi, 2011; Chérel et al., 2014; Anschütz et al., 2014). More than 50 enzymes are activated by K^+ which cannot substitute by Na^+; further, protein synthesis requires high K^+ concentration (Tester and Davenport, 2003). Supply of K^+ fertilizers ameliorates detrimental effects of salinity on plant performance (Cakmak, 2005; Shabala and Pottosin, 2014).

A strong positive correlation between root's K^+ retention ability and salt tolerance was revealed in many species (Cuin et al., 2012; Chen et al., 2007b,c,d; Smethurst et al., 2008; Sun et al., 2009). Here we provide compelling evidence that K^+ retention in the photosynthetic-active mesophyll tissue may be an equally important trait contributing to the overall salinity tolerance (Fig. 3). The R^2 value for group 1 plants (include tolerant and intermediate genotypes; Fig. 3C) was as high as 0.43, suggesting that over 40% of genetic variability in salinity tolerance in this cluster was attributed to just one physiological trait, namely better cytosolic K^+ retention. Thus, targeting this trait in the

breeding programs may open additional avenues in pyramiding the overall tolerance. The reported results may also explain previous reports of higher shoot (or leaf) K^+ content found under salinity stress in salt tolerant compared with sensitive varieties in barley (Liang, 1999), tomato (Taleisnik and Grunberg, 1994; Al-Karaki, 2000), soybean (Essa, 2002), and lucerne (Smethurst et al., 2008).

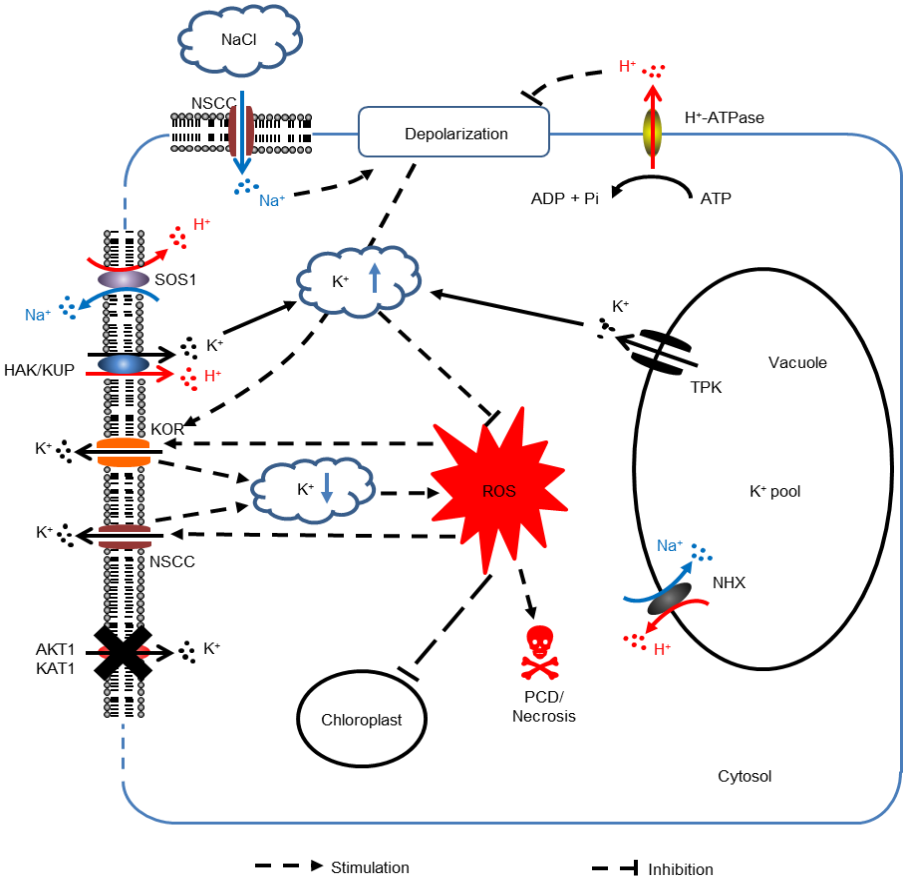


Fig. 7. The suggested model depicting ionic mechanisms contributing to K^+ retention in leaf mesophyll in barley. Upon NaCl exposure, the plasma membrane is depolarized by the massive entry of external Na^+ via non-selective cation channel (NSCC) and results in K^+ loss mediated by depolarization-activated K^+ outward rectifying channel (KOR) channels. Cytosolic K^+ homeostasis is disrupted. The accompanied production of ROS has detrimental effects of leaf photochemistry in chloroplasts and, in severe cases, could trigger PCD or lead to necrosis. ROS also activate NSCC exacerbating K^+ loss from cytosol. Compared with salt sensitive genotypes, higher vacuolar K^+ pool in the leaf mesophyll in salt tolerant barley genotypes allows plants to maintain cytosolic K^+ homeostasis by releasing vacuolar K^+ into cytosol; this process is mediated by tonoplast K^+-permeable channels (TPK in the model). It is also suggested that tolerant varieties may have higher Na^+/H^+ NHX exchanger activity and, thus, can replace vacuolar K^+ by Na^+ to maintain its osmotic pressure in vacuole. As K^+ uptake by AKT1 into leaf mesophyll is inhibited by the membrane depolarization, plants should rely on high affinity K^+ uptake to restore cytosolic K^+ homeostasis (a HAK/KUP family K^+/H^+ co-transporter in the model). A concurrent K^+ and H^+ uptake through HAK/KUP exchanger results in a +2 net charge transfer and may further depolarise the plasma membrane, resulting in a futile cycle of K^+. This process is partially compensated by higher ATPase-driven H^+-pumping activity in sensitive varieties.

In our electrophysiological experiments, salinity stress was applied to leaf segments of plants grown under *control* conditions and, thus, bypassed all possible uncertainties and confounding factors related to genotypic differences in plants' ability to exclude Na^+ from uptake or load it into the xylem. Interestingly, sensitive and tolerant barley varieties showed a different extent of dependence on their ability to retain K^+ in leaf mesophyll (Fig. 3C), indicated by different slope of relationship between the damage index and net K^+ efflux. As commented above, the physiological rationale behind this observation may be that tolerance varieties may possess some other (additional) mechanisms to deal with salinity (such as higher SOS1 or NHX activity; Fig. 7), while sensitive varieties must rely heavily on K^+ retention (e.g. tissue tolerance) for their survival.

Reflecting a true tissue tolerance mechanism, mesophyll K^+ retention may be targeted in the breeding programs using the protocols described in this work (Fig. 2). Varieties identified in this work as possessing superior (YWHKSL, DYSYH, Numar) and poor K^+ retention under salinity stress (Aizao 3; Mundah; Keel) can be used to produce double haploid populations to fine-map QTLs conferring tissue tolerance mechanisms in barley. This trait may be then added to more traditional (previously identified) traits to create salt-tolerant varieties via "pyramiding" approach (Flowers and Yeo, 1995; Shabala, 2013). In the past, such work was done predominantly for QTLs related to Na^+ exclusion traits. Selected examples include mapping *Nax1* and *Nax2* genes in durum wheat (Lindsay et al., 2004; James et al., 2006a; Munns et al., 2012) and *HvNax3* and *HvNax4* genes in barley (Shavrukov et al., 2010; Rivandi et al., 2011). Also, *HKT2;1/2*-like, *HKT2;3/4*-like, *HKT1;1/2*-like, *HKT1;3*-like, *HKT1;4*-like, and *HKT1;5*-like genes (contributing to Na^+ accumulation in the shoot) were mapped to the wheat–barley chromosome groups 7, 7, 2, 6, 2, and 4, respectively (Huang et al., 2008). However, until now no QTLs related to K^+ retention trait have been reported in barley and wheat. The present work provides a possibility to overcome this limitation and exploit QTLs associated with K^+ retention in leaf mesophyll.

4.3.2 Salt tolerant genotypes have intrinsically higher leaf K^+ content

Barley varieties screened in this work varied largely in their damage index (Fig. 1A), NaCl-induced K^+ efflux from the leaf mesophyll (Fig. 3A), and initial leaf K^+ status (Fig. 4A). The fact that more tolerant plants contained intrinsically higher K^+ levels in their leaves (Fig. 4A) may be indicative of several possible mechanisms involved (Fig. 7). First, these plants may simply have higher capacity for K^+ uptake (e.g. higher HAK/KUP

activity; Fig. 7) and thus accumulated more K^+ under non-stress conditions. Cytosolic K^+ content is maintained at roughly the same level in all species (Leigh, 2001, Dreyer and Uozumi, 2011); thus the difference between genotypes will be most likely in the size of the vacuolar K^+ pool. When exposed to salinity, all barley plants will start to lose K^+ from leaf mesophyll (Fig. 3A). As this process is detrimental to normal cell metabolism, K^+ leaked from the cytosol to apoplastic space will be quickly (within 10-15 min; Shabala et al., 2006) replaced on the expense of the vacuolar K^+ pool. Even assuming that sensitive and tolerant varieties do not differ in their ability to control any other transport systems (which is not necessarily the case; see below), the larger the vacuolar K^+ pool, the longer plants will be able to maintain the mesophyll cells' viability (hence, lower damage index). Indeed, under 200 mM NaCl treatment, K^+ activity in vacuole and cytosol was decreased respectively from 235 to 150 mM and 79 to 64 mM in barley leaf mesophyll (Cuin et al., 2003), suggesting the possibility of releasing vacuolar K^+ to cytosol to compensate cytosolic K^+ loss under salt stress. This might explain the existence of a weak but significant positive correlation between initial leaf K^+ content and the relative FW (Fig 4B) reported in our work. These results are consistent with data reported by Ren et al. (2005) in rice who showed that with higher initial shoot K^+ content, a NIL(*SKC1*) line which had better ability to maintain higher K^+/Na^+ ratio showed significantly higher K^+ content after salinity stress than its isogenic control.

Another contributing factor is a better ability of NaCl-challenged mesophyll cells to maintain plasma membrane potential. Indeed, both K^+ uptake and efflux in leaf cells are mediated by an array of voltage-gated inward- (e.g. AKT1; KAT) and outward- (GORK) rectifying potassium-selective channels (Shabala, 2003; Véry and Sentenac, 2003; Chérel et al., 2014). As the voltage-current relationship for these channels is highly non-linear, even a small (e.g. 10 mV) difference in membrane potential may result in a several fold difference in net K^+ flux along the cellular membrane (Chen et al., 2007c). The reported significant negative correlation between leaf K^+ content and NaCl-induced K^+ efflux from mesophyll (Fig. 4C) is consistent with this suggestion.

Maintaining K^+ homeostasis in cytosol of leaf mesophyll would help to provide conditions for photosynthesis under salinity stress and thus ensure plants' life. High K^+ content is also essential to reduce the amount of reactive oxygen species (ROS) in photosynthetically active mesophyll tissue (Cakmak, 2005). ROS are one of the molecules that signal the low K^+ status in plants (Ashley et al., 2006), and high levels of ROS are detrimental to cellular structures, causing either programmed cell death (PCD) or

necrosis (Breusegem and Dat, 2006). K^+ loss per se has also been suggested to be a critical step in PCD (Peters and Chin, 2007). Ion disequilibrium-induced PCD was shown to occur in yeast and *Arabidopsis* roots under salinity stress (Huh et al., 2002). K^+ retention seems to play a critical role in preventing salinity stress-induced PCD. Expression of animal CED-9 anti-apoptotic gene in tobacco leaf mesophyll has resulted in improved plant phenotype under saline conditions and was linked with better K^+ retention in mesophyll (Shabala et al., 2007b), and the number of cells undergoing PCD in Arabidopsis *gork1-1*mutants plants lacking functional outward-rectifying K^+ channels was ten-fold lower compared with wild type (Demidchik et al., 2010). The mechanism beyond is that ROS-activated K^+ efflux through GORK channels results in dramatic K^+ loss from plant cells, which stimulates proteases and endonucleases, and promotes PCD (Demidchik et al., 2014). ROS were also shown to be able to activate K^+-permeable non-selective cation channels (NSCC) (Demidchik and Maathuis, 2007). Altogether, 40 different NSCC have been found in *Arabidopsis* genome (Davenport, 2002); these may differ dramatically in their gating properties. Patch-clamp studies have provided explicit evidences that some of NSCC can be activated by various ROS species such as H_2O_2 or $OH^{\bullet}$ (Demidchik et al., 2003; Demidchik and Maathuis, 2007; Zepeda-Jazo et al., 2011). Thus, it may be suggested that the difference between sensitive and tolerant varieties may be attributed to the difference in a population of NSCC and their sensitivity to ROS. This possibility is depicted in Fig. 7. The ability of plants to prevent salt stress-induced ROS production may also contribute to this difference. From this point of view, the better balancing of cytosolic K^+ through releasing vacuolar K^+ may reduce the production of ROS and thus prevent or delay the commencement of necrosis or PCD process in leaf mesophyll leading to better performance of the plant under salinity stress (Fig. 7).

4.3.3 H^+ efflux as a compensatory mechanism under salinity stress

It was shown before that K^+ retention ability in roots correlates positively with the maintenance of negative membrane potential (Chen et al., 2007c, barley) and higher H^+ efflux (Maksimović et al., 2013, barley; Sun et al., 2009, poplar). Plant plasma membrane H^+-ATPase is an electrogenic enzyme, which belongs to the P type family of cation-translocating pumps, and builds a membrane potential and generates the proton-motive force that drives nutrient uptake across the plasma membrane (Palmgren, 2001; Pedersen et al., 2012). H^+-ATPase mRNA accumulated in roots of *Atriplex nummularia* and tobacco culture cells under saline stress (Morsomme and Boutry, 2000), and a salt-

tolerant barley varieties had 2.5-3 fold higher intrinsic level of H^+-ATPase activity compared with sensitive ones in their roots (Chen et al., 2007c).

In the present study, NaCl-induced H^+ flux from leaf mesophyll varied between different barley varieties (Fig. 5A) but was correlated *negatively* with salinity tolerance (Fig. 5B). This is opposite to previous reports on barley roots (Chen et al., 2007c). Thus, it appears that the role of H^+-ATPase and the pattern of its induction in root and leaf tissue differ dramatically, at least in barley. More studies are needed to explicitly explain this difference. At the moment, several possible mechanisms should be considered. First, the positive correlation between net K^+ and H^+ flux may be a part of the energy-saving strategy in salt tolerant varieties. While indeed increased H^+-ATPase pumping activity is instrumental in restoring membrane potential and closing GORK-like channels (thus preventing or significantly reducing K^+ loss from the cytosol; Chen et al. 2007c; Shabala and Cuin, 2008), this strategy is energy-demanding and draws upon available ATP pool. Moreover, it was shown that salt-sensitive barley varieties rely heavily on organic osmolytes for their osmotic adjustment to salt, while tolerant varieties use mainly inorganic ions for these purposes (Chen et al., 2007b). Thus, it can be hypothesised that salt-sensitive varieties may need more ATP to not only fuel plasma membrane H^+-ATPase pumps to restore the membrane potential but also maintain other important metabolic processes (e.g. *de novo* synthesis of compatible solutes) to survive under saline conditions. Second, it could be envisaged that sensitive varieties have less capacity to control net Na^+ uptake and thus undergo higher membrane depolarisation. If this is the case, they will then need to have higher rate of H^+-pumping to restore membrane potential and bring it to the level of tolerant varieties. Third, under depolarization conditions of salinity passive K^+ uptake via AKT1 or KAT (Dennison et al., 2001; Pilot et al., 2003; Boscari et al., 2009) channels becomes thermodynamically not possible. Given the fact that sensitive varieties have intrinsically less amounts of K^+ in their tissues (Fig. 4B) and, thus, are limited in their ability to maintain cytosolic K^+ homeostasis on expense of vacuolar pool, the only remaining option is to rely on high-affinity K^+ uptake. In leaf mesophyll, such uptake is mediate by the members of the HAK/KUP transporter family (Rubio et al., 2000) which co-transport K^+ with H^+. Thus, higher net H^+ efflux in sensitive varieties may be essential to drive high-affinity K^+ uptake aimed to restore cytosolic K^+ homeostasis under salinity stress. Also, a concurrent K^+ and H^+ uptake through HAK/KUP exchanger results in a net charge transfer of +2 which and may further depolarise the plasma membrane, resulting in a futile cycle of K^+. Higher ATPase-driven

H^+-pumping may partially compensate this process. All these options (summarised in Fig. 7) need to be evaluated in direct experiments.

4.4 Materials and methods

4.4.1 Plant material and growth for electrophysiological experiments

46 barley (44 *Hordeum vulgare* L.; and two *Hordeum vulgare* ssp. *Spontaneum*, CPI 11284-48 and SYR 01) varieties were used in this study. Seeds were obtained from multiples sources and multiplied in our laboratory. Five varieties were sowed every day in 2 L pots (6 plants per pot) filled with the standard potting mix (Chen et al., 2007d). Plants were grown in December 2012 using the University of Tasmania glasshouse facilities essentially as described in our early publications (Chen et al., 2007d). Plants were irrigated with tap water by automatically watering system three times per day. Two to 3-week old seedlings were used for non-invasive microelectrode ion flux (MIFE) measurements.

4.4.2 Estimation of biomass and the damage index

Suppl. Fig. S2. Examples illustrating quantification of salinity damage to plants by scoring of the damage index (1 to 10 scale). Plants were grown in glasshouse and treated with 300 mM NaCl for 40 days. (A) Salt tolerant variety SYR 01; (B) intermediate salt tolerant variety Lixi 143; (C) salt sensitive variety Unicorn.

Fresh weight (FW) and dry weight were measured in glasshouse experiments. Similar to the above, plants were grown in Jan-Feb 2013 in the glasshouse facilities at the University of Tasmania. Twelve to 14 seeds for each variety were sown in a 4.5 L PVC pot filled with the standard potting mix. Seedling numbers were thinned to eight uniformed in each pot just before salinity treatment. Experiments were performed in triplicates. Plants were irrigated twice per day by an automatic watering system with dripper outlets for both control condition and salinity treatment. A saucer was placed under each pot. Salinity treatment (300 mM NaCl in irrigation solution) was started when

seedlings were five days old and lasted for 40 days. The damage index of each variety was scored 1 to 10 based on its performance under salinity treatment before harvesting. The higher damage index score represents the lower tolerance. An example of scoring the damage index is shown in Suppl. Fig. S2. A collective sample of the shoot biomass for all plants in each pot (eight in total) was taken. The shoot was cut 1 cm above the potting mix and its fresh weight was recorded immediately. Samples were then dried at 65 ^{0}C in a Unitherm Dryer (Birmingham, UK) for 72 h and the dry weight was recorded. Shoot water content (SWC) was calculated based on the difference between fresh and dry weight.

4.4.3 K^+ content estimation

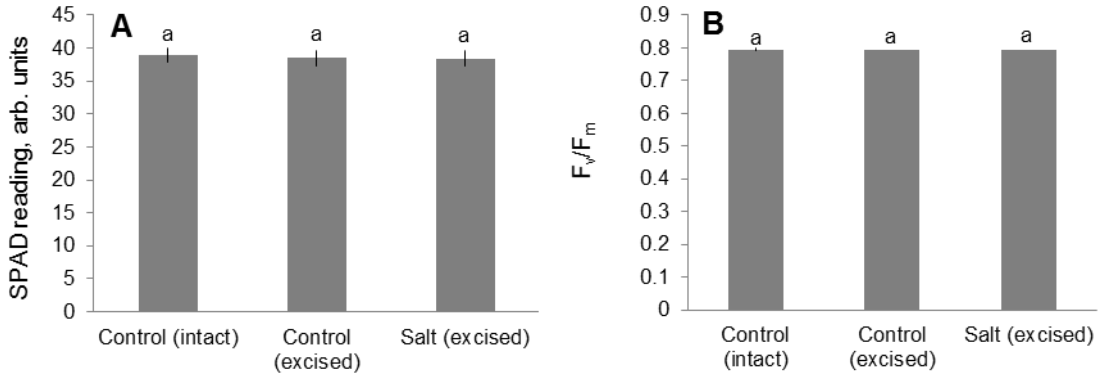
A 0.1 g of ground dry barley leaves was weighed (GR-120, A&D Company Ltd, Japan) and digested in 5 mL 70% HNO_3 and 2 mL 30% H_2O_2 in a 120 mL Teflon digestion vessel in a microwave digester (MDS-2000 microwave digestion system, CEM Corporation, USA) for 1 h. The digested solution was then transferred to a 15 mL centrifuge tubes, diluted with distilled water to a final volume of 15 mL and centrifuged at 5000 g for 10 min at room temperature (Avanti J-30I centrifuge, Beckman Coulter, Germany). 0.2 mL of the supernatant was diluted to a final volume of 10 mL and K^+ content was measured using a Flame Photometer (PFP7, Jenway, UK).

4.4.4 Non-invasive ion flux estimation (MIFE) measurements

Net ion fluxes (K^+ and H^+) were measured using MIFE (microelectrode ion flux estimation) technique (Shabala et al. 2006). Shortly, borosilicate glass capillaries (GC150-10; Clark Electrochemical instruments, Pangbourne, Berks, UK) were pulled out using a vertical puller. The pulled electrodes were then dried in an oven at 225ºC overnight, and silanized with tributilchlorosilane (Cat. No. 90796; Fluka, Busch, Switzerland). After drying and cooling, electrodes were back filled with backfilling solutions (15 mM NaCl + 40 mM KH_2PO_4; pH 6.0 adjusted using NaOH for H^+ and 200 mM KCl for K^+) and tips of respective electrodes were front filled with commercially available selectophore cocktails (K^+, Cat. No. 60031; H^+, Cat. No. 95297, Sigma). Prepared ion selective microelectrodes were calibrated in two set of respective standards (with and without 100 mM NaCl added), before and after use. Only electrodes with a slope above 50 mV per decade and correlation 0.999 or higher were used.

4.4.5 Screening mesophyll K^+ retention ability by non-invasive MIFE technique

The overall experimental procedure for screening mesophyll K^+ retention ability by the MIFE technique is illustrated in Fig.1. Plants were grown in a glasshouse (Fig. 2A). Small leaf segments (approx. 5× 8 mm) were cut from the middle part of the second leaf (7 to 10 d old) exposing leaf mesophyll tissue for measuring net fluxes of K^+ and H^+ (Fig. 2B). Cut segments were immediately floated on the surface of the Basic Salt Media solution (BSM: 0.1 mM $CaCl_2$ and 0.5 mM KCl, pH 5.7 non-buffered) (Fig. 2C) and kept overnight in the darkness to minimize possible confounding effects of tissue damage (see Shabala and Newman 1999 for justification and details). This procedure had no negative impact on leaf physiological characteristics and photochemistry, as judged by the absence of any significant changes in leaf chlorophyll content of chlorophyll fluorescence characteristics (Suppl. Fig. S3 A, B).



Suppl. Fig. S3. Chlorophyll content (A) and chlorophyll fluorscence F_v/F_m ratio (B) of excised leaf segments floated after 10 h (overnight in darkness) in BSM solution, and after 30 min of 100 mM NaCl treatment. Mean ± SE (n=7).

Prepared samples were immobilized in the Perspex holder and placed in a chamber containing 100 mM NaCl + BSM solution for 0.5 h salinity pre-treatment (Fig. 2D). K^+ and H^+ electrodes were positioned next to exposed leaf mesophyll under x100 microscopic magnification, ~40 μm above the mesophyll tissue (Fig. 2E and F). During measurements, electrodes were moved by a computer-controlled hydraulic manipulator between two position (40 and 110 μm above the leaf surface), in a 12-s square-wave cycle. Steady state NaCl-induced K^+ efflux and H^+ flux were recorded and displayed on a screen (Fig. 2G). Fluxes were measured for 5 minutes. The recorded voltage outputs of the electrodes were converted into net fluxes and expressed in $nmol^{-2}$ s^{-1} using MIFEFLUX software (Newman, 2001; Shabala et al., 2006). The interpreted values of specific ion fluxes of the output results from MIFE measurements were shown in a excel format and plotted figure after averaging 6-8 replicates (Fig. 2H and I).

4.4.6 Chlorophyll content and chlorophyll fluorescence measurements

Leaf chlorophyll content and maximal photochemical efficiency of photosystem II (chlorophyll fluorescent F_v/F_m value) were measured in methodological experiments from both intact leaves and excised leaf segments floated on the surface of the BSM solution overnight, as descried above. These measurements were conducted using SPAD-520 chlorophyll meter (Konica Minolta, Japan) and OS-30p chlorophyll fluorometer (Opti-sciences, USA), respectively.

4.4.7 Statistical analysis

All data (given as mean ± SE, n = sample size) were analysed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). All of the replicates are biological replicates. The significance of the correlation between different parameters was determined by Bivariate Correlations based on Pearson Correlation (2-tailed). The data used for correlation analysis are the average values of measured independent parameters for each variety. Significance between different treatments was determined by one-way ANOVA based on Duncan's multiple range test. Same lowercase letters mean no significant difference.

Chapter 5

Durum and bread wheat differ in their ability to retain potassium in leaf mesophyll: implications for salinity stress tolerance[#]

Abstract

Understanding the intrinsic mechanisms involved in the differential salinity tolerance between bread and durum wheat is essential for breeding salt-tolerant varieties to cope with the global salinity issue threatening future food supply. In the past, higher salinity tolerance in bread compared with durum wheat has been attributed to its better ability to exclude Na^+ from uptake. Here we show that another mechanism, namely more superior K^+ retention ability in the leaf mesophyll, also contributes to this difference. A strong positive correlation ($r = 0.64$, $P < 0.001$) was found between NaCl-induced K^+ efflux in leaf mesophyll and an overall salinity tolerance in 48 wheat varieties. However, while the above correlation was strong in bread wheat, it was statistically insignificant in durum wheat. Consistent with these findings, significantly higher relative leaf K^+ content was found in bread than durum wheat. Contrary to root tissues, the role of voltage-gated K^+ channels in retention in wheat mesophyll was relatively small, and non-selective cation channels played a major role in controlling intracellular K^+ homeostasis. Moreover, a significant negative correlation between NaCl-induced mesophyll H^+ flux and mesophyll K^+ retention was found and interpreted as a compensatory mechanism employed by sensitive varieties to regain K^+ leaked into the apoplast. It is concluded that bread and durum wheat show different strategies of coping with salinity, and that targeting mechanisms conferring K^+ retention in leaf mesophyll may be a promising way to improve the overall salinity tolerance in these species.

Keywords

Cytosolic potassium homeostasis, plasma membrane, ion channel, ion flux, ROS, depolarization

[#], This chapter has been published as: Wu H, Shabala L, Zhou M, Shabala S (2014) Plant & Cell Physiology 55: 1749–1762.

Abbreviations

BSM, basic salt media; DPI, diphenylene iodonium; Gd^{3+}, gadolinium chloride; HKT, high affinity K^+ transporter; GORK/KOR, K^+ outward rectifying channel; MIFE, microelectrode ion flux estimation; NSCC, nonselective cation channel; QTL, quantitative trait loci; ROS, reactive oxygen species; SWC, shoot water content; TEA^+, tetraethylammonium chloride

5.1 Introduction

Wheat is one of the major food crop species that provides nearly 55% of the carbohydrates consumed worldwide (Gupta et al., 1999). About 95% of the wheat grown worldwide is hexaploid bread wheat, with most of the remaining 5% being tetraplopid durum wheat (Shewry, 2009). In many sub-continent (e.g. India, Pakistan) and Middle East region (Iran, Egypt, Libya) wheat producing countries up to 10% of all wheat belt is affected by salinity (Colmer et al., 2006), while in some places such as Western Australia more than half of wheat farms are suffering from salinisation problem. Thus, in the light of predicted population growth to 9.3Bln by 2050 (Lee, 2011), improving salinity tolerance in wheat is an urgent task to cope with the possible shortage of food supply in the near future.

Bread wheat is known to possess higher salt tolerance compared with durum wheat (Munns and Tester, 2008). This difference was previously attributed mainly to the better ability of bread wheat cultivars to exclude Na^+ from uptake (Shah et al., 1987; Gorham 1990; Colmer et al., 2006; Cuin et al., 2010; Munns et al., 2012). As a result, bread wheat accumulates less Na^+ in the shoot (relative to durum wheat) and thus maintains higher K^+/Na^+ ratio in leaves (Gorham et al., 1987; Gorham, 1990; Dvořák et al., 1994; Munns et al., 2003; Lindsay et al., 2004); a trait that is considered to be most essential for salinity stress tolerance. However, maintaining high K^+/Na^+ ratios in photosynthetically-active mesophyll cells can be achieved not only by stronger Na^+ exclusion but also by improved K^+ retention in leaf mesophyll. Indeed, bread wheat showed not only lower shoot/leaf Na^+ content but also higher K^+ content compared with durum wheat (Shah et al., 1987, Gorham, 1990). However, to the best of our knowledge none of published papers addressed the issue of mechanisms underlying this difference, largely due to the lack of suitable techniques. Indeed, in most cases the authors analysed Na^+ and K^+ content in the

entire bulk of the leaf or shoot (Mass and Poss, 1989; Munns et al., 2000; Sairam et al., 2002; Husain et al., 2003; Poustini and Siosemardeh, 2004; El-Hendawy et al., 2005; Cuin et al., 2010, 2012) using either AAS, ICP or flame photometry and were therefore unable to differentiate between ion accumulation patterns in various tissues and intracellular compartments. Several more advanced studies used cryo-scanning electron microscopy (SEM) X-ray microanalysis to quantify cellular and subcellular distribution of Na^+ and K^+ in various leaf tissues (James et al., 2006b; Husain et al., 2004). However, these papers did not address the issue of specific membrane transporters contributing to reported difference in K^+ content between different tissues and/or compartments. It remains unclear whether higher K^+ content in leaf mesophyll under saline conditions originate from higher rate of K^+ uptake by roots, or is it due to higher xylem K^+ loading and its transport to the shoot, or whether higher K^+ content is associated with better K^+ retention by leaf mesophyll.

In our recent preliminary work, we have compared kinetics of net NaCl-induced K^+ fluxes from the mesophyll tissue of four contrasting (two bread and two durum) wheat varieties (Wu et al., 2013) and reported a significant correlation between the ability of plants to retain K^+ in leaf mesophyll after exposure to salinity, and the overall plant salinity tolerance. Here we have extended this work to undertake a large-scale validation of the essentiality of K^+ retention in leaf mesophyll as a factor differentiating the extent of salinity tolerance between durum and bread wheat species. A strong positive correlation ($r = 0.64$, $P < 0.001$) between the ability of leaf mesophyll to retain K^+ and the overall salinity tolerance (estimated as a damage index) was found while screening a large number (~ 50) of wheat genotypes. K^+ retention ability in the leaf mesophyll (measured as the magnitude on net K^+ flux in response to an acute salt treatment) was found to be significantly higher (1.7 folds difference) in bread than in durum wheat; also higher was the relative leaf K^+ content in bread wheat in plants grown under saline conditions. We further discuss the molecular nature of ion transporters mediating K^+ retention in leaf mesophyll and the prospects of using this trait in wheat breeding for salinity stress tolerance.

5.2 Results

5.2.1 Genetic variability in salinity tolerance in wheat

Salinity stress (300 mM NaCl treatment given to pot-grown plants for ~ six weeks) severely affected plant growth, with relative fresh weight (FW) of salt treated plants being

below 20% of that in control (Fig. 1A). A seven- fold difference was observed in the relative FW values of all 48 wheat varieties screened (Fig. 1A), with bread wheat varieties consistently outperforming durum ones (the difference significant at $P < 0.001$; Fig 1B). Also significant was the difference in the salinity tolerance between bread and durum wheat varieties quantified by the damage index (Fig. 1C). In bread wheat, the mean damage index was 3.55 ± 0.22, while in durum wheat the average value was 6.38 ± 0.21 (significant at $P < 0.001$; Fig. 1D). A strong positive correlation (r = 0.79, $P < 0.001$) between the extent of plant salt tolerance (estimated as the damage index) and relative FW under saline conditions was found (Fig. 1E) suggesting that each of these agronomical indices can be used as a measure of salinity tolerance in wheat.

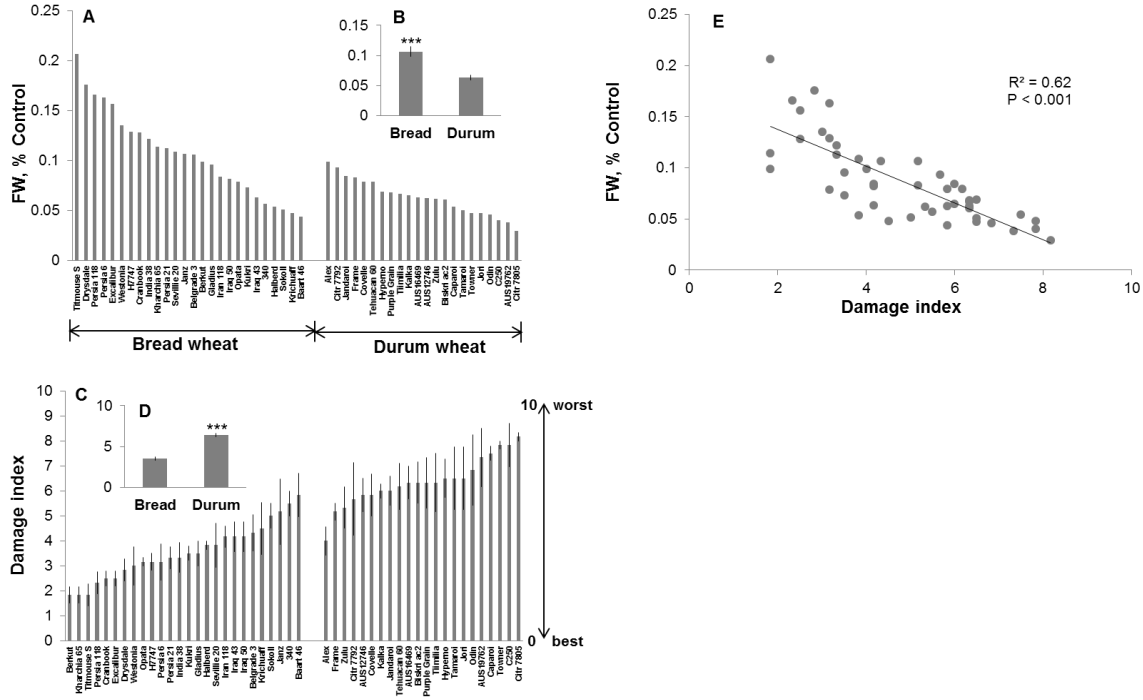


Fig. 1. Genetic variability of salinity tolerance in wheat. (A) Relative fresh weight (FW) of bread and durum wheat under salt stress. Plants were treated with 300 mM NaCl for 38 days under glasshouse conditions. (B) Pooled mean values of relative FW values for all bread wheat and durum wheat genotypes. (C) Damage index of bread wheat and durum wheat under salt stress on 0 to 10 scale (0 – no impact of salt stress; 10 (worst) – plants are dead). Mean ± SE, n=24 (3 pots x 8 plants in each); (D) Pooled mean values of damage index for all bread and durum wheat genotypes. (E) Correlation between relative FW and damage index. Each point represents a separate variety.

Shoot water content (SWC) ranged from 90% to 60.3% in salt-grown plants compared to controls (Fig. 2A). Bread wheat genotypes were outperforming durum ones in their ability to maintain water in the shoot (90-81.7% vs 83.4-60.3% range; significant at P < 0.001; Fig. 2AB). A strong and significant correlation between the relative SWC and a damage index (r = 0.89, $P < 0.001$) was found. Taken together, the above results suggest

that (1) a large variability in salinity stress tolerance exists among wheat accessions, and (2) bread wheat is indeed much more tolerant compared with durum wheat.

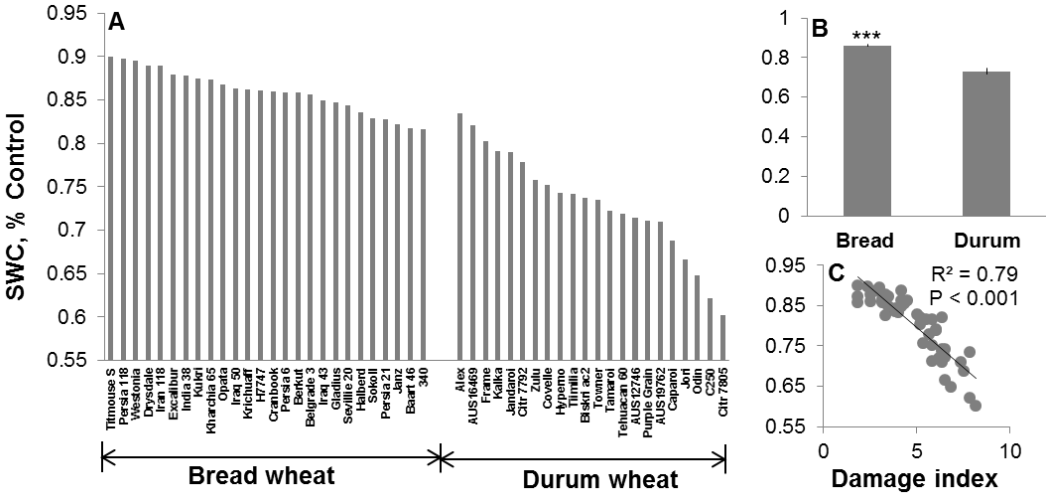


Fig. 2. Genetic variability in shoot water content (SWC) in wheat under salt stress. (A) Relative SWC of bread wheat and durum wheat. Plants were treated with 300 mM NaCl for 38 days under glasshouse conditions. (B) Pooled mean values of RWC for all bread and durum wheat genotypes. (C) Correlation between relative SWC and damage index. Each point represents a separate variety.

5.2.2 K^+ retention in leaf mesophyll is correlated with salinity tolerance

Adding 100 mM NaCl to leaf mesophyll of control-grown plants (mimicking NaCl accumulation in leaf apoplast under saline conditions) resulted in an immediate and rapid net K^+ efflux from measured leaf segments (Fig. 3A). Given the fact that electrodes were positioned above the mesophyll tissue under strong (x200 times) microscope magnification, and mesophyll and vascular bundle cells can be easily differentiated by their steady-state fluxes (Shabala et al., 2002), this NaCl-induced K^+ efflux was interpreted as originating predominantly from the mesophyll cells. The extent of this efflux showed a seven-fold variability among 48 varieties screened (Fig. 3B), with average K^+ efflux ranging from -1517 (highest) to -215 (lowest) nmol m^{-2} s^{-1} (Fig. 3B). On average, K^+ loss from durum wheat mesophyll was twice higher compared with a similar loss from bread wheat (-457 ± 27 vs -811 ± 55 nmol m^{-2} s^{-1}, respectively; significant at $P < 0.001$; Fig 3C). A strong and significant negative correlation was found between leaf K^+ retention in MIFE experiments and plant damage index under saline conditions in glasshouse experiments ($r = 0.64$, $P < 0.001$; Fig. 3D). Also significant was a correlation between K^+ retention and SWC ($r = 0.71$, $P < 0.001$; Fig. 3E).

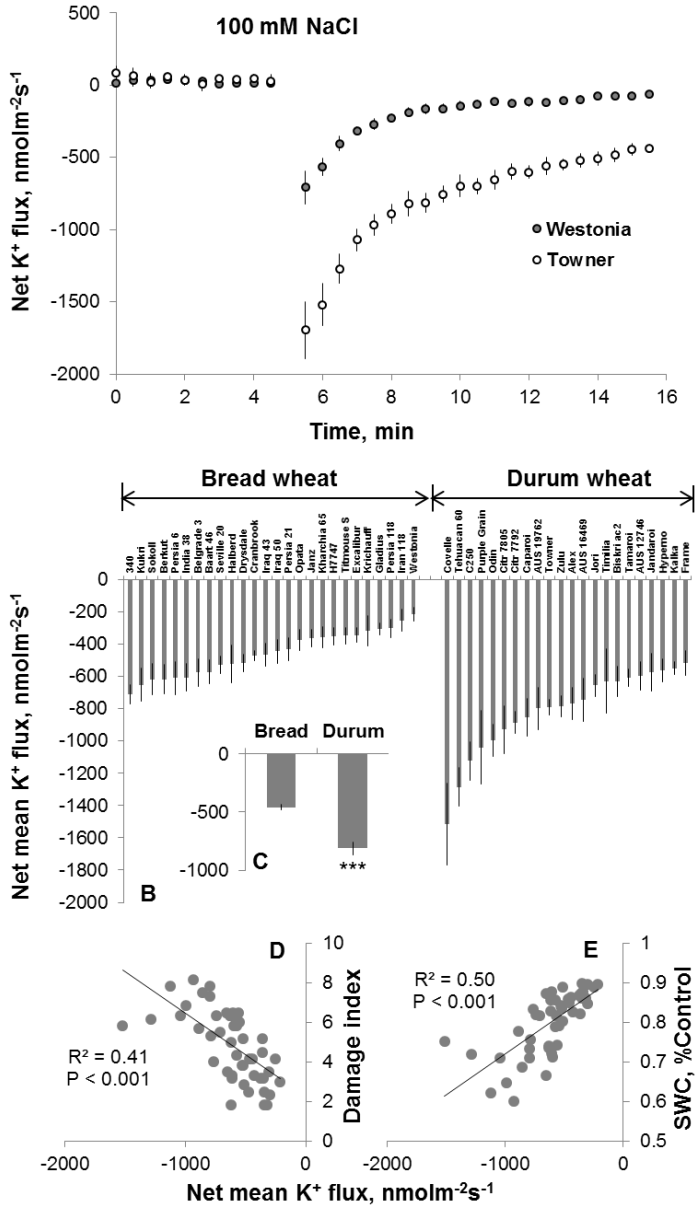


Fig. 3. Genetic variability in NaCl-induced K^+ efflux from leaf mesophyll and its correlation with salinity tolerance in wheat. (A) Representative examples of transient NaCl-induced net K^+ flux from leaf mesophyll in bread (cv Westonia) and durum (cv Towner) wheat. Mean ± SE (n= 6). (B) Average K^+ efflux values from leaf mesophyll in response to 100 mM NaCl from a range of bread and durum wheat cultivars. Mean ± SE (n= 5−13). (C) Pooled mean values of average K^+ efflux for all bread and durum wheat genotypes. (D, E) Correlation between net NaCl-induced K^+ flux from leaf mesophyll and damage index (D) and SWC (E). Each point represents a separate variety.

5.2.3 NaCl-induced K^+ efflux in leaf mesophyll was significantly supressed by both TEA^+ and Gd^{3+}

A rapid net H^+ efflux was triggered from leaf mesophyll by 100 mM NaCl treatment (Fig. 4A). With a single exception for variety Hyperno, all other 47 varieties showed NaCl-induced average H^+ efflux from the leaf mesophyll (Fig. 4A). Similar to K^+, net H^+ fluxes varied greatly between the varieties ranging from -215 nmol m^{-2} s^{-1} (highest; in Covelle) to -6 nmol m^{-2} s^{-1} (lowest; in Kalka) (Fig. 4B). No significant difference (at $P < 0.05$) was found between durum and bread wheat for NaCl-induced average net mesophyll H^+ flux (Fig. 4C). A weak but significant positive correlation ($r = 0.35$, $P < 0.05$) was found between NaCl-induced net H^+ and K^+ fluxes from leaf mesophyll (Fig. 4D).

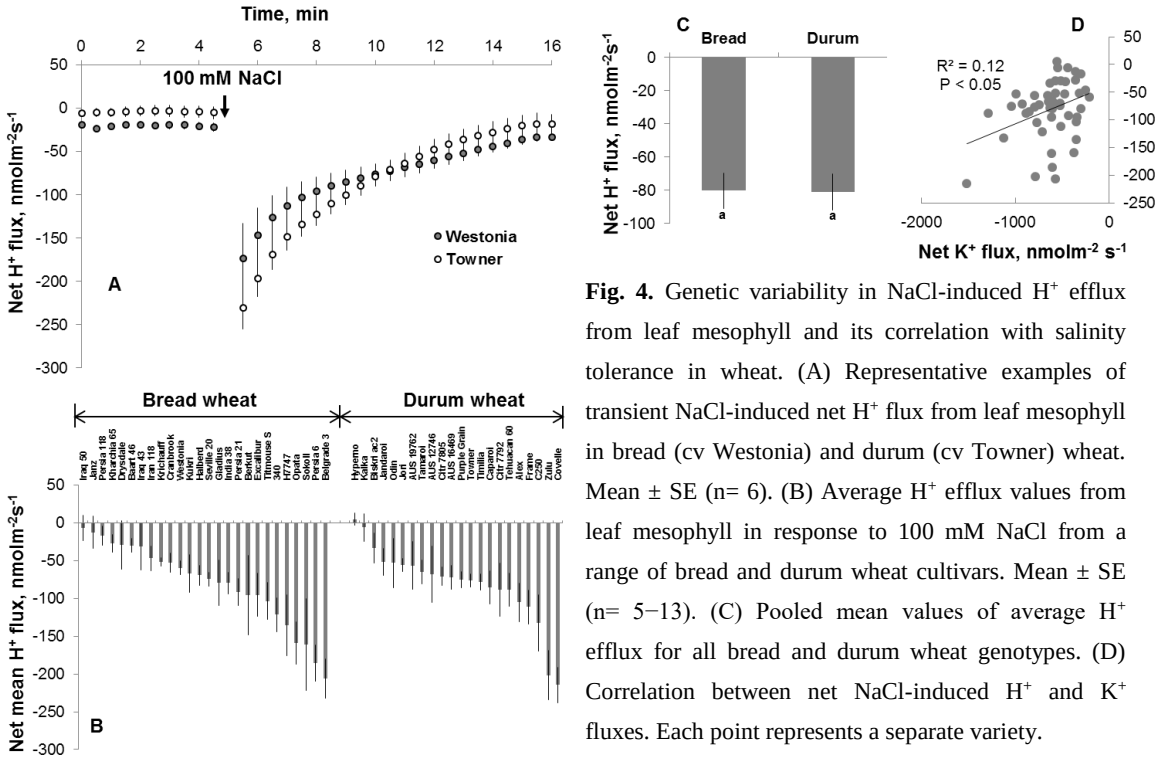


Fig. 4. Genetic variability in NaCl-induced H^+ efflux from leaf mesophyll and its correlation with salinity tolerance in wheat. (A) Representative examples of transient NaCl-induced net H^+ flux from leaf mesophyll in bread (cv Westonia) and durum (cv Towner) wheat. Mean ± SE (n= 6). (B) Average H^+ efflux values from leaf mesophyll in response to 100 mM NaCl from a range of bread and durum wheat cultivars. Mean ± SE (n= 5−13). (C) Pooled mean values of average H^+ efflux for all bread and durum wheat genotypes. (D) Correlation between net NaCl-induced H^+ and K^+ fluxes. Each point represents a separate variety.

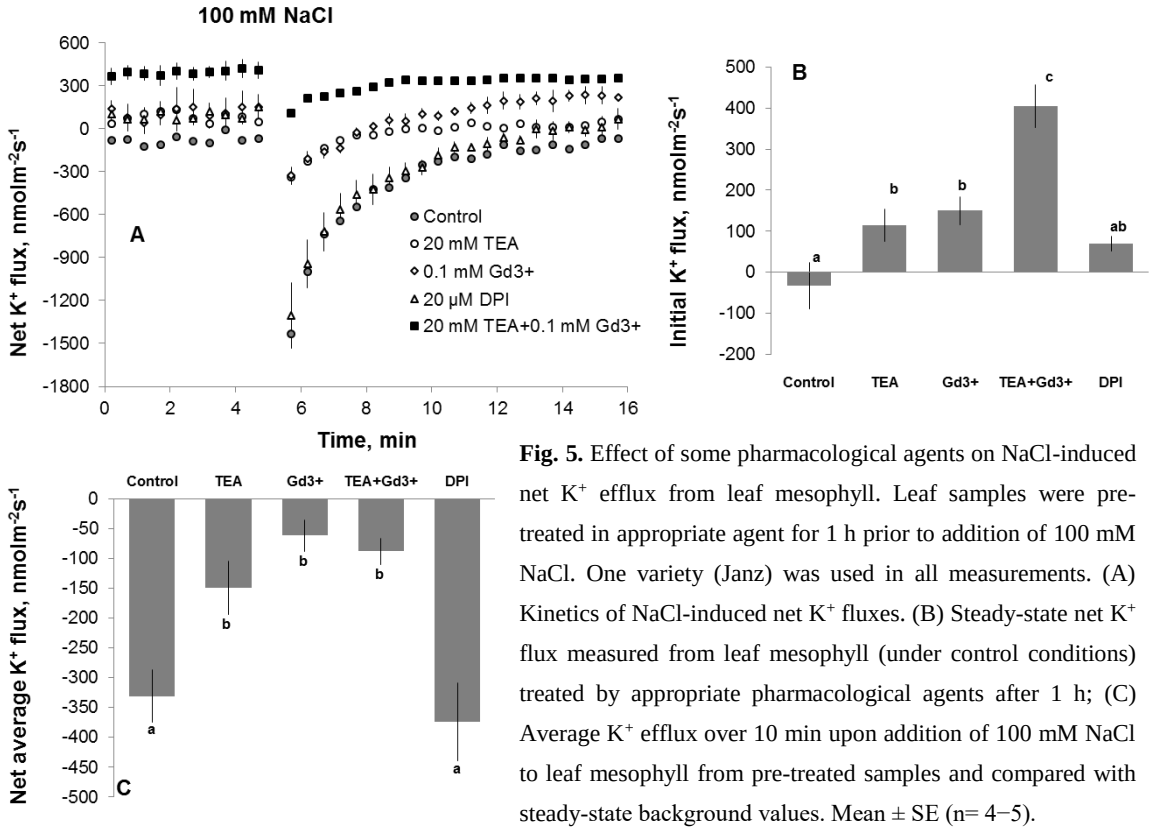


Fig. 5. Effect of some pharmacological agents on NaCl-induced net K^+ efflux from leaf mesophyll. Leaf samples were pre-treated in appropriate agent for 1 h prior to addition of 100 mM NaCl. One variety (Janz) was used in all measurements. (A) Kinetics of NaCl-induced net K^+ fluxes. (B) Steady-state net K^+ flux measured from leaf mesophyll (under control conditions) treated by appropriate pharmacological agents after 1 h; (C) Average K^+ efflux over 10 min upon addition of 100 mM NaCl to leaf mesophyll from pre-treated samples and compared with steady-state background values. Mean ± SE (n= 4−5).

NaCl-induced K^+ efflux was inhibited by both 20 mM TEA^+ (tetraethylammonium chloride, a known blocker of K^+ selective channels), and 0.1 mM Gd^{3+} (gadolinium

chloride, a known blocker of non-selective cation channels, NSCC) but not by 20 μM DPI (diphenylene iodonium, a known inhibitor of NADPH oxidase) (Fig. 5A). With an exception of DPI pre-treatment, all other treatments (e.g. TEA^+, Gd^{3+}, or their combined application) resulted in a significantly higher initial K^+ flux from leaf mesophyll compared with control (Fig. 5B). TEA blocked 55% of NaCl-induced K^+ efflux, while 81% inhibition was found in the samples pre-treated with Gd^{3+} (Fig. 5C).

5.2.4 The relationship between leaf K^+ status and mesophyll K^+ retention and overall salinity tolerance

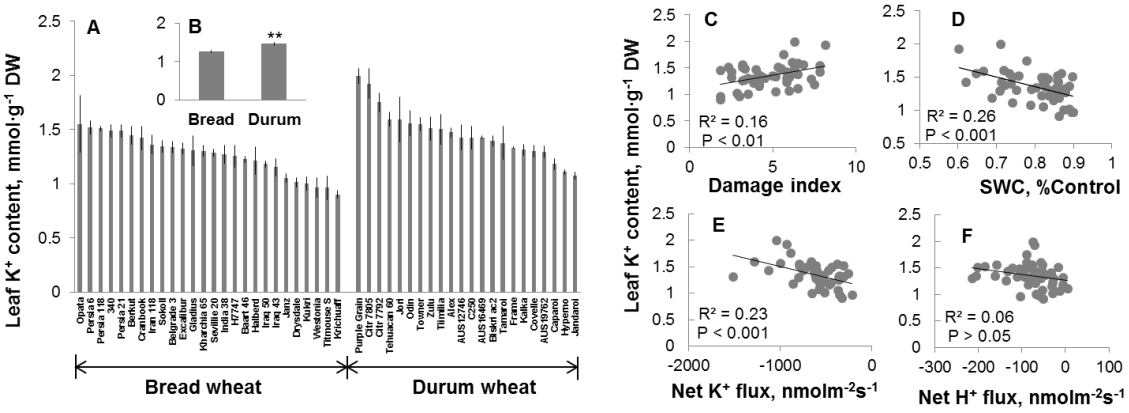


Fig. 6. Genetic variability in leaf K^+ content in wheat. (A) Intrinsic variation in leaf K^+ concentration in bread and durum wheat plants grown under control condition. Mean ± SE (n=3 (3 pots with eight plants in each)). (B) Pooled mean values of leaf K^+ content for all bread and durum wheat genotypes shown in A. (C - F) Correlation between leaf K^+ concentration in control-grown plants and salinity-induced damage index (C), SWC of salt-grown plants (D), the peak average NaCl-induced K^+ (E) and H^+ (F) flux from leaf mesophyll in wheat. Each point represents a separate variety.

Total leaf K^+ content varied broadly in wheat varieties under control conditions, ranging from the highest 1.99 $mmol^{-1}$ g^{-1} DW (durum wheat Purple Grain) to the lowest 0.90 $mmol^{-1}$ g^{-1} DW (bread wheat Krichuaff). Higher leaf K^+ content was found in durum wheat (1.46 ± 0.05) than in bread wheat (1.26 ± 0.04) (Fig. 6B; significant at $P < 0.001$). This result is consistent with previous findings showing that salt tolerance in wheat was associated with lower but not higher K^+ content in plants under control condition (Cuin et al. 2010). In agreement with this a significant negative correlation was found between leaf K^+ content under control condition and the overall salinity tolerance ($r = 0.40$, $P < 0.01$; Fig. 6C; $r = 0.51$, $P < 0.001$; Fig. 6D) and K^+ retention in leaf mesophyll ($r = 0.48$, $P < 0.001$; Fig. 6E). No or weak correlation ($r = 0.24$, $P > 0.05$; Fig. 6F) was found between leaf K^+ content under control condition and NaCl-induced H^+ flux in leaf mesophyll. In

contrast to previous finding in barley (Wu et al., 2015d), higher initial leaf K^+ content in wheat showed no benefit to overall salinity tolerance (Fig. 6).

Compared with seven-fold difference in both relative FW (Fig. 1A) and NaCl-induced K^+ efflux (Fig. 3B), relative leaf K^+ content of salt-grown plants showed only a three-fold variability, ranging from 1.05 (highest; in bread wheat Krichuaff) to 0.35 (lowest; in durum wheat Citr 7805) (Fig. 7A). On average, bread wheat leaves maintained more K^+ under salt stress than those of durum wheat (0.80 ± 0.03 vs 0.68 ± 0.04; significant at $P < 0.01$; Fig 7B). This is consistent with the evidence of stronger ability of bread wheat to retain K^+ in leaf mesophyll shown in Fig. 3BC. A significant positive correlation was found between relative leaf K^+ content and K^+ retention in leaf mesophyll ($r = 0.37$, $P < 0.05$; Fig. 7C), and relative SWC ($r = 0.36$, $P < 0.05$; Fig. 7F). At the same time relative leaf K^+ content correlated negatively with the NaCl-induced net H^+ flux ($r = 0.35$, $P < 0.05$; Fig 7D) and the damage index ($r = 0.40$, $P < 0.01$; Fig. 7E).

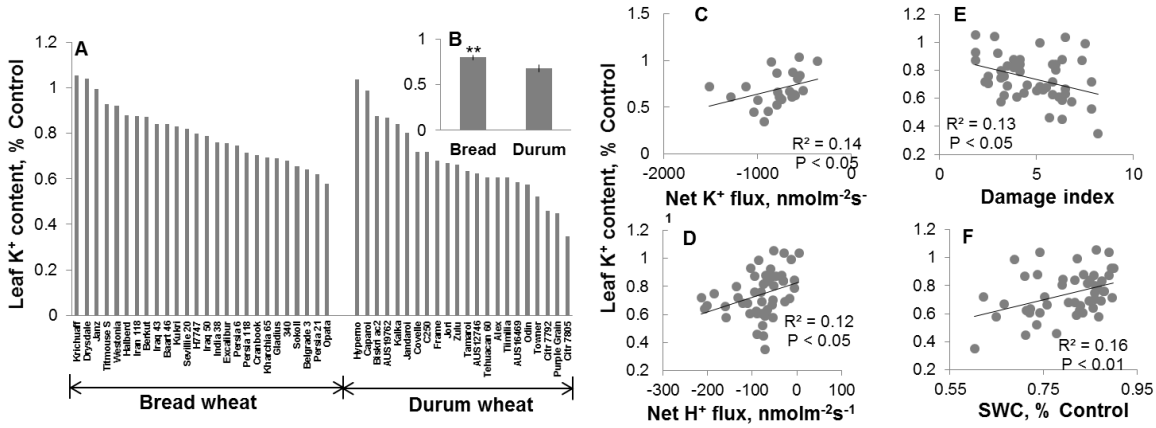


Fig. 7. Genetic variability in salinity-induced changes in leaf K^+ content in wheat. Data shows leaf K^+ concentration in plants grown at 300 mM NaCl. mM NaCl for 38 days and expressed as % of K^+ concentration in control. (A) Mean relative K^+ content in different bread and durum wheat cultivars. Mean ± SE (n= 3 (3 pots with eight plants in each). (B) Pooled mean values of relative leaf K^+ content in salt-grown bread and durum wheat genotypes shown in A. (C - F) Correlation between relative leaf K^+ concentration and the average NaCl-induced K^+ (C) and H^+ (D) flux from leaf mesophyll, salinity-induced damage index (E), and SWC of salt-grown plants (F) in wheat. Each point represents a separate variety.

5.2.5 Correlation between salinity tolerance and K^+ retention in leaf mesophyll is essential for bread but not durum wheat

While leaf K^+ retention has significantly correlated with salinity tolerance in all 48 wheat varieties (Fig. 3), this correlation was driven essentially by the bread wheat (Fig. 7). While analysed separately, a significant negative correlation between salinity tolerance and K^+ retention in leaf mesophyll was found in bread wheat ($r = 0.41$, $P < 0.05$; Fig. 8A) but not durum wheat ($r = 0.17$, $P > 0.05$; Fig. 8B). Also a weak relationship

between mesophyll K^+ retention ability and SWC in saline-grown plants was found in durum wheat (r = 0.33, $P > 0.05$; Fig. 8D), while in bread wheat this correlation was strong and statistically significant (r = 0.48, $P < 0.01$; Fig. 8C).

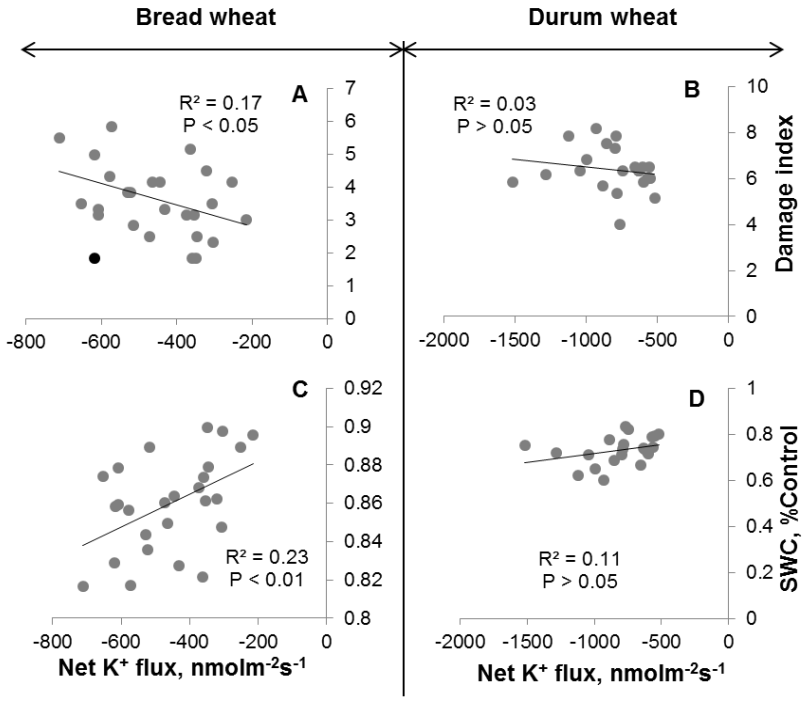


Fig. 8. Correlation between the average NaCl-induced net K^+ efflux from leaf mesophyll and damage index (A, B) and SWC (C, D) analysed separately bread (A, C) and durum (B, D) wheat species. Each point represents a separate variety.

5.3 Discussion

5.3.1 K^+ retention is essential to maintain higher K^+/Na^+ ratio in bread wheat

Similar to other species, salinity tolerance in wheat was always associated with higher tissue K^+/Na^+ ratio (Dubcovsky et al., 1995; Ashraf and Khanum, 1997; Munns et al., 2000), and this ratio was higher in bread compared with durum wheat (Gorham et al., 1987; Dvořák et al., 1994; Dubcovsky et al., 1995). As the bread wheat was also shown to have higher rate of Na^+ exclusion from uptake (Dvořák and Gorham, 1992; Davenport et al., 1997; Cuin et al., 2009), it is not surprising that the higher K^+/Na^+ shoot ratio and the overall better salinity tolerance in bread compared with durum wheat was attributed to such Na^+ exclusion. In this work we show that the above mechanism is only one of several possible mechanisms determining this trait, and that optimal K^+/Na^+ ratio in the shoot may also be determined by the superior ability of the plant to retain K^+ in leaf mesophyll. Although a positive correlation between salinity stress tolerance and shoot K^+ content was observed in many species (e.g. wheat - El-Hendawy et al. 2005; tomato - Taleisnik and Grunberg, 1994; Al-Karaki, 2000; soybean - Läuchli and Wieneke, 1979, Essa, 2002; Lucerne - Smethurst et al., 2008; barley - Liang et al., 1999), none of these works investigated the mechanisms behind this high shoot K^+ content in tolerant cultivars.

Here we show that K^+ retention in mesophyll is one of the key mechanisms contributing to this trait.

Potassium is recognized as a rate-limiting factor for crop yield (Maathuis and Amtmann, 1999; Pettigrew, 2008; Dreyer and Uozumi, 2011). Potassium concentration in soil is around 0.1 − 1 mM (Wang and Wu, 2013) while optimal K^+ content in plants is more than 100-fold higher (Dreyer and Uozumi, 2011; Wang and Wu, 2013). Over 80 metabolic enzymes are activated by K^+ (Evans and Sorger, 1966; Suelter, 1970) and, thus, require high cytosolic K^+ content. Under salinity stress plants suffer from K^+ deficiency (Grattan and Grieve, 1992), and K^+ content of the shoot tissue can be reduced by several folds (Muralitharan et al., 1992). Supplying K^+ fertilizers can ameliorate detrimental effects of salinity on plant performance (Cakmak, 2005; Shabala and Pottosin, 2014).

Due to high K^+ mobility in the phloem (van Goor and van Lune, 1980; Marschner et al., 1996), a very substantial part of K^+ (up to 80% in some species; Jeschke and Pate, 1991; Marschner et al., 1996) delivered to the shoot by the transpiration stream is returning back to the root. While this process plays an important role in controlling root K^+ acquisition (Marschner et al., 1996), it may also potentially lead to a futile cycle. We believe this may be a case in wheat grown under saline conditions. In this context, sensitive varieties lacking efficient K^+ retention in mesophyll will release more K^+ in the apoplast; this K^+ will then be returned to the root. This will be "interpreted" by the root as a signal to reduce K^+ acquisition (Marschner et al., 1996; Ahmad and Maathuis, 2014; Anschütz et al., 2014), exacerbating K^+ deficiency and arresting plant growth and cell metabolism, with the consequent penalties for yield.

5.3.2 Targeting K^+ retention in leaf mesophyll: an emerged opportunity for breeders?

Until now, genetic improvement of salinity tolerance in wheat was focused largely on Na^+ exclusion trait (Munns et al., 2002; Munns et al., 2006; Munns et al., 2012). Progress, however, is still much less than one would hope. Recently, Munns et al. (2012) have shown that the presence of *TmHKT1;5-A* gene (*Nax2* locus), encoding a Na^+-selective transporter located on the plasma membrane of root cells surrounding xylem vessels, increased durum wheat grain yield by 25% compared to near-isogenic lines without *Nax2*. Unfortunately, despite this improvement, the grain yield of transformed plants under saline conditions was still only 50% of plants grown under non-saline conditions. We have recently argued that the overall progress may be stronger if Na^+ exclusion traits are

complemented by tissue tolerance traits (Shabala, 2013). One of components of the tissue tolerance mechanism is K^+ retention in the cytosol. Indeed, in addition to being a crucial macronutrient as discussed above, potassium is also playing a major signalling role in plant adaptive responses (Anschütz et al., 2014). Two specific aspects deserve a separate comment. Salinity tissue tolerance relies strongly on plant's ability to sequester Na^+ in leaf vacuoles and, in addition to tonoplast Na^+/H^+ exchangers, relies heavily on vacuolar H^+- pumps (Apse et al., 1999; Shabala, 2013). At the same time, cell ATP pool is expected to be significantly reduced under saline conditions, largely due to increased need for *de novo* synthesis of compatible solutes (Chen et al., 2007b). This leaves H^+-PPase as the major driver for energising Na^+/H^+ exchanger activity. However, vacuolar H^+-PPase activity is known to be K^+ dependent (Rea and Poole 1993; Serrano et al. 2007), implying that the failure to retain K^+ in mesophyll cell will compromise leaf tissue tolerance. Indeed, a strong negative correlation between NaCl-induced K^+ efflux from mesophyll and overall salinity tolerance (Fig. 3) is in a full agreement with this notion. Another important aspect is essentiality of high cytosolic K^+ in preventing stress-induced programmed cell death (Shabala et al., 2007a; Shabala and Pottosin, 2014).

In our view, the next logical step would be to undertake a QTL mapping for genes conferring mesophyll K^+ retention trait. In this work we have identified varieties with superior (Westonia and Persia 118 for bread wheat, Kalka and Hyperno for durum wheat) and poor (340 and Sokoll for bread wheat, C250 and Odin for durum wheat) K^+ retention in leaf mesophyll under salinity stress. These varieties can be used as the potential parent lines to create a double haploid population and fine-map related QTLs. Then existing Na^+ exclusion and (newly discovered) K^+ retention traits may be pyramided to create salt tolerant wheat genotypes. As discussed in section 3.4.3 in Chapter 3, the MIFE technique-based measure of NaCl-induced K^+ efflux from leaf mesophyll (mesophyll K^+ retention ability) was recommended remains the most preferred current method, the feasibility of using simple K^+ concentration measurements are worth a proper investigation. Furthermore, although a strong positive correlation ($r = 0.71$, $P < 0.001$; Fig. 3E) was found between relative SWC and meshoyll K^+ retention under salt stress, the relative SWC still was not recommended to be used for substituting for osmotic adjustment since osmotic adjustment is executed not only by ions (e.g. Na^+, K^+, and Cl^- etc.) but also by compatible solutes such as proline, betaine.

5.3.3 Identity of ion transport systems conferring K^+ retention in leaf mesophyll

A specific mechanism underlying K^+ efflux from wheat mesophyll remains largely unknown. By analogy with other plant systems, two possible candidates should be considered. One of them is K^+ outward rectifying channel (KOR), and another one - non-selective cation channel (NSCC). In barley roots, NaCl-induced K^+ efflux was shown to be mainly mediated by depolarization activated KOR channels (Chen et al., 2007c) and strongly correlated with the extent of NaCl-induced depolarization of epidermal root cells. However, transient NaCl-induced K^+ fluxes from wheat roots were only ~ 10% of those measured from barley (Cuin et al., 2008, 2010) and not related to membrane depolarisation. A recent comparison of barley and pea roots has also shown that in the latter species, K^+ leak from the root is mediated predominantly by NSCC activated by stress-induced accumulation of reactive oxygen species (Bose et al., 2014a). We believe that the similar scenario may be applicable to wheat leaf mesophyll. Two lines of evidence support this statement. First, effect of NSCC channel blocker, Gd^{3+}, was stronger than that of TEA (KOR channel blocker) under both control (Fig. 5B) and salt treatment (Fig. 5C). Second, in this work we report a significant *negative* correlation between the extent of NaCl-induced K^+ flux from mesophyll and NaCl-activation of H^+-efflux (Fig. 4C, D). Given the fact that this NaCl-induced H^+ extrusion is vanadate-sensitive (Kerkeb et al., 2001; Wu et al., 2013) and, thus, most likely originates from the salinity-induced activation of the plasma membrane H^+-ATPase, the above negative correlation may be suggestive that K^+ efflux from wheat mesophyll is largely independent on the extent of plasma membrane depolarisation. Instead, the observed activation of H^+ efflux (Fig. 4) by salinity may be interpreted as a compensatory mechanism involved in an attempt to regain K^+ already leaked into apoplast. However, as H^+ extrusion comes with energy cost (e.g. ATP to fuel H^+ pump), higher rate of pumping in sensitive varieties may be an additional factor reducing available ATP pool and shunting down metabolic and growth processes in these genotypes.

Interestingly, in contrast to K^+ efflux inhibition by TEA and Gd^{3+}, no effect of DPI (an inhibitor of NADPH oxidase), was found on K^+ efflux from wheat leaf mesophyll (Fig. 5). This suggests that NADPH oxidase is not likely to be a major source of ROS production in salt-treated wheat mesophyll tissues, and that other subcellular compartment(s) such as chloroplasts and/or mitochondria (Apel and Hirt, 2004; Peshev et al., 2013; Pottosin et al., 2014b; Bose et al., 2014b) play more central role in this process. In this context, patch-clamp experiments on Arabidopsis roots showed that TEA-sensitive depolarization-activated outward rectifying K^+ channels were strongly activated by

hydroxyl radicals, OH• (Demidchik et al., 2010). OH• is known to be most reactive (toxic) of all ROS species (Halliwell and Cross, 1994; Apel and Hirt, 2004), and salinity-induced increase in tissue OH• is widely reported (Bose et al., 2014b; Pottosin et al., 2014b). These finding may explain some beneficial effect (~50% inhibition; Fig. 5) of TEA on salinity-induced K^+ efflux from wheat mesophyll.

In conclusion, our results strongly suggest that K^+ retention in leaf mesophyll is an essential mechanism employed by bread wheat to deal with the detrimental effects of salinity. It also appears that the role of voltage-gated K^+ channels in retention in wheat mesophyll is relatively small, and ROS-activated NSCC are most likely pathways for the observed K^+ leak under saline conditions. This hypothesis has to be tested in direct patch-clamp experiments in the future.

5.4 Materials and methods

5.4.1 Plant materials and growth conditions

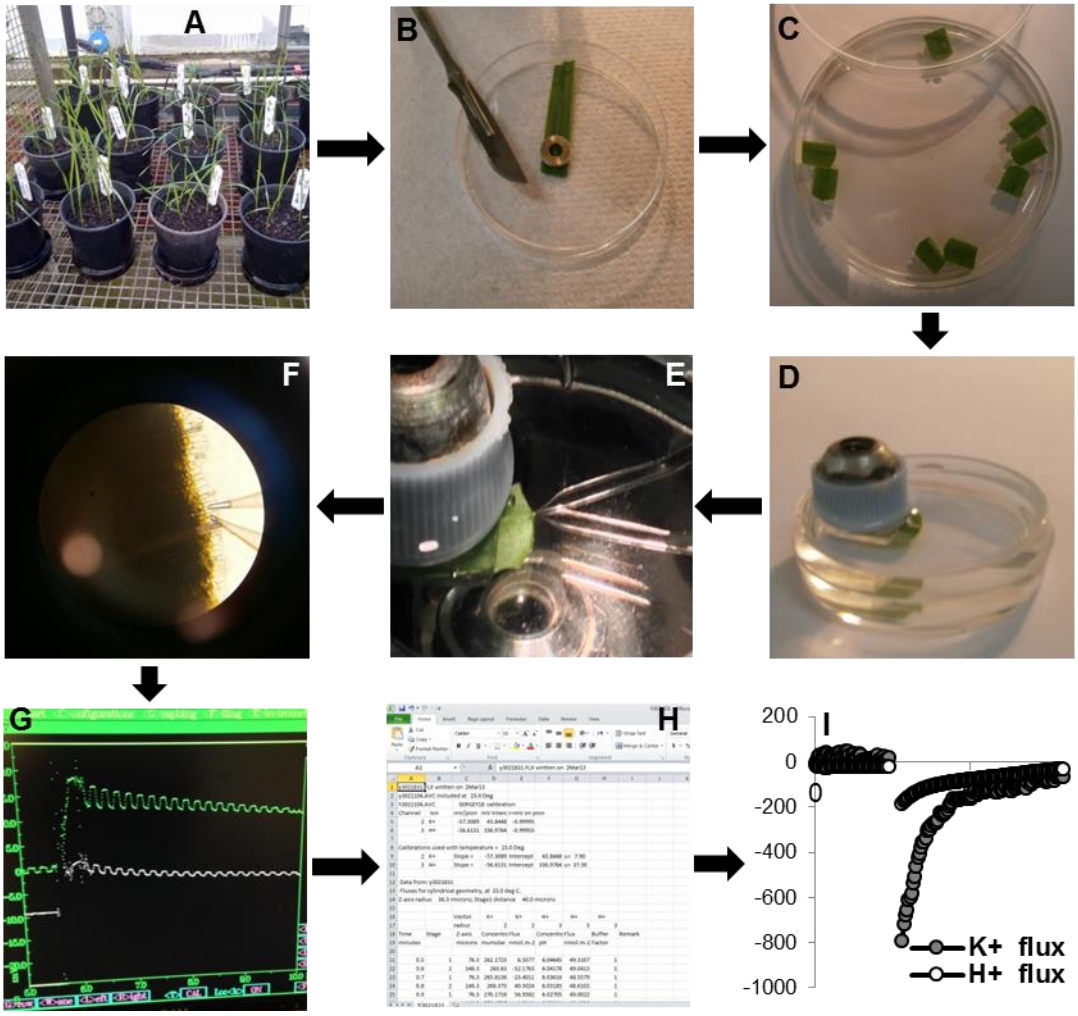
26 bread wheat (*Triticum aestivum*) and 22 durum wheat (*Triticum turgidum* spp. *durum*) were used in this study. All seeds were obtained from different sources and multiplied in our laboratory. Plants were grown in 2 L pots (6 plants per pot) filled with the fertilised standard potting mix in March-April 2013 using glasshouse facilities at the University of Tasmania essentially as described in Chen et al. (2007d). Plants were irrigated with tap water by automatic watering system three times per day. Leaves from two- to three- week old plants grown under control conditions were used for non-invasive microelectrode ion flux estimation measurements.

5.4.2 Preparation of ion-selective microelectrodes for non-invasive ion flux measurements

Net NaCl-induced K^+ and H^+ fluxes from leaf mesophyll were measured by using non-invasive microelectrode ion flux estimation (the MIFE; Shabala et al., 2006) technique (also known in the literature as NMT). Briefly, borosilicate glass capillaries (GC 150-10; Harvard Apparatus, Kent, UK) were pulled to make the blank microelectrodes. These blanks were then oven-dried at 225 ºC overnight and silanized with tributylchlorosilane (No 282707, Sigma-Aldrich, St. Luis, MO, USA). After drying and cooling, silanized microelectrodes were filled with backfilling solutions (15 mM NaCl + 40 mM KH_2PO_4; pH 6.0 adjusted using NaOH for H^+ and 200 mM KCl for K^+), and the tip of the microelectrodes were then filled with commercially available ionophore cocktails (K^+

60031, H^+ 95297; both from Sigma-Aldrich, St. Luis, MO, USA). Finally, all prepared ion selective microelectrodes were calibrated in a set of appropriate standards before and after use. Only microelectrodes with a slope of above 50 mV per decade and correlation of 0.999 or better were used.

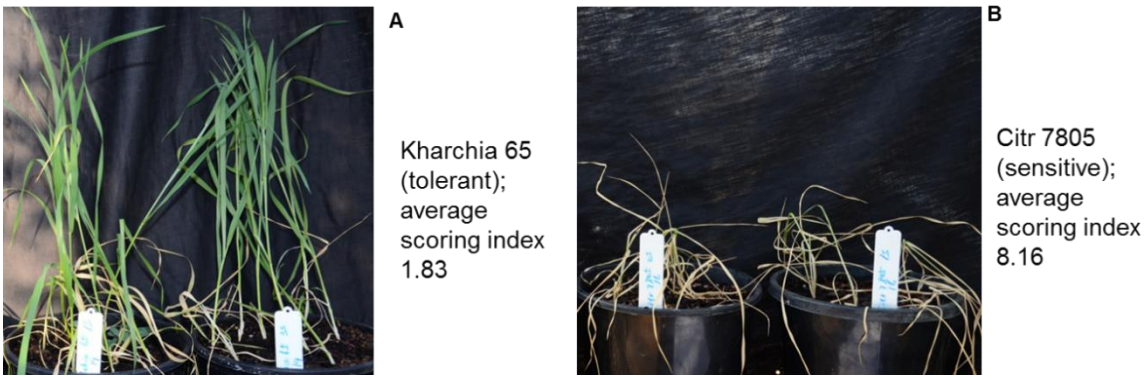
5.4.3 Screening K^+ retention in leaf mesophyll in wheat by the MIFE technique



Suppl. Fig. S1. The procedure of screening wheat varieties for their ability to retain K^+ in leaf mesophyll using the MIFE technique. (A) Plants were grown in glasshouse until two to three weeks old; (B) One of the leaves was excised, and small leaf segments cut; (C) Samples were left floating on the surface of the BSM solution in a plastic Petri dish and kept in the darkness overnight; (D) Leaf samples were mounted in a Perspex and placed (submerged) in a Petri dish filled with BSM solution; (E) Ion selective microelectrodes were positioned 40 ìm from the exposed mesophyll tissue on a cross-cut section; (F) During measurements microelectrodes were moved between two positions, 40 and 110 µm from the mesophyll, by a computer-controlled hydraulic manipulator in a 12-s square-wave cycle; (G) K^+ and H^+ electrode outputs were recorded and displayed on screen; (H) Using electrode calibration characteristics, measured electrical signals were converted into net fluxes (in nmol m^{-2} s^{-1}) and tabulated in an Excel-compatible format; (I) recorded NaCl-induced K^+ efflux and H^+ flux were plotted as figures.

In this work we developed a rapid screening method to assess a large number of wheat genotypes (48 varieties) for salinity tolerance based on the MIFE technique by measuring NaCl-induced K^+ efflux in leaf mesophyll. Plants were grown in a glasshouse (Suppl. Fig. S1A) and 7–10 days old leaves were excised and used in experiments. Leaves were cut to small cross-sectional segments using a sharp blade (Suppl. Fig. S1B). Leaf segments were placed in a Petri dish containing basic salt media (BSM, 0.1 mM $CaCl_2$ and 0.5 mM KCl; pH 5.7 non-buffered) (Suppl. Fig. S1C) and kept floating on the surface in darkness overnight to minimize possible confounding effects of tissue damage on ion fluxes (Shabala and Newman, 1999).

For MIFE experiments a leaf segment was mounted in a Perspex holder which was placed in a measuring chamber containing BSM solution and left for 40 min adaptation (Suppl. Fig. S1D). Tips of K^+ and H^+ selective microelectrodes were placed within a few micrometres one from another (Suppl. Fig. S1E), and located 40 μm away from the exposed leaf mesophyll using an inverted microscope (Suppl. Fig. S1F). During measurements microelectrodes were moved between two positions, 40 and 110 μm from the mesophyll, by a computer-controlled hydraulic manipulator in a 12-s square-wave cycle. NaCl-induced fluxes were recorded for 10 min from each of 48 wheat varieties screened. The recorded voltage outputs of the ion selective microelectrodes were calculated based on the recorded electrical potential using cylindrical diffusion geometry, and were converted into net ion fluxes using calibration values of the electrodes using MIFEFLUX software (Newman, 2001; Shabala et al., 2006). Suppl. Fig. S1G shows a computer screen with recorded ion fluxes. The values of ion fluxes (the output results) from the MIFE measurements were analysed in Excel format (Suppl. Fig. S1H,I).



Suppl. Fig. S2. Quantification of impact of salinity stress on plant performance by scoring of the damage index (0 to 10 scale). 0 – no visual symptoms of damage; 10 – plants are dead. Plants were grown in glasshouse under long term salt treatment (300 mM NaCl for 38 days). Two examples are show: (A) Salt tolerant bread variety Kharchia 65 showing only mildly signs of salt stress (average scoring index 1.83), and (B) salt sensitive durum variety Citr 7805, with most leaves being dead (average scoring index 8.16).

5.4.4 Glasshouse experiments

Effect of salinity on plant fresh (FW) and dry (DW) weight was investigated in the glasshouse experiments. Plants were grown in the glasshouse facilities at the University of Tasmania. 12 to 14 seeds of each variety were sown in a 4.5 L PVC pot using the standard potting mix (in triplicates). After emerging of the seedlings (roughly 6 days later) the plant numbers in each pot were uniformly thinned to eight plants, and salt treatment (300 mM NaCl) was applied for 38 days. A saucer was placed under each pot, and plants were irrigated twice per day by an automatic watering system with dripper outlets for both control condition and salinity treatment. The damage index (quantified on 0 to 10 scale and based on the extent of leaf chlorosis and plant survival rate; Zhou et al. 2012) was scored immediately before harvesting (Suppl. Fig. S2). The higher damage index score represents the lower tolerance. During harvest, the stem was cut 1 cm above the potting mix and shoot FW was measured immediately. A collective sample of the shoot biomass for all plants in each pot (eight plants in total) was taken. Samples were then dried at 65 ^{0}C for 72 h in a Unitherm Dryer (Birmingham, UK) and shoot DW was measured. Shoot water content (SWC) was calculated based on the relative difference between the fresh weight and the dry weight.

5.4.5 Measuring leaf K^+ content

0.1 g of ground dry wheat leaves was used for ion extraction. Dried samples were mixed with 5 mL 70% HNO_3 and 2 mL 30% H_2O_2 and digested in a 120 mL Teflon digestion vessel using a microwave digester (MDS-2000 microwave digestion system,

CEM Corporation, USA). The digested solution was transferred to a 15 mL centrifuge tube and toped up with distilled water to a final volume of 15 mL. The prepared samples were centrifuged at 5 000 g for 10 min at room temperature (Avanti J-30I centrifuge, Beckman Coulter, Germany). A volume of 0.2 mL of the centrifuged digested solution was diluted with distilled water to a final volume of 10 mL and used for measuring K^+ content with a Flame Photometer (PFP7, Jenway, UK).

5.4.6 Pharmacological experiments

Mechanisms underlying NaCl-induced K^+ efflux in leaf mesophyll were investigated further through a series of pharmacological experiments using one variety (bread wheat Janz). Leaf segments prepared as described above for MIFE measurements were pre-treated for 1 h with one of: 20 mM tetraethylammonium chloride (TEA^+, a known blocker of K^+ selective channels), 0.1 mM gadolinium chloride (Gd^{3+}, a known blocker of NSCC channels), and 20 μM diphenylene iodonium (DPI, a known inhibitor of NADPH oxidase), before starting MIFE measurements. Salinity treatment (100 mM NaCl) was given after recording initial ion fluxes for 5 min. All chemicals are from Sigma-Aldrich, St, Louis, MO.

5.4.7 Statistical analysis

All data (given as mean ± SE, n = sample size) were analysed by using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). All of the replicates are biological replicates. Comparison of different parameters between bread wheat and durum wheat were done by independent samples t-test. The symbol "**" states for $P<0.01$, and "***" states for $P<0.001$. The significance of correlations between different parameters was determined by Bivariate Correlations based on Pearson Correlation (2-tailed). The data used for correlation analysis are the average values of measured independent parameters for each variety. Significance between different pharmacological treatments was determined by one-way ANOVA based on Duncan's multiple range test. Different lower case letters represent the significant differences while the same lowercase letters state for no significant difference.

Chapter 6

Developing and validating a high-througput assay for salinity tissue tolerance in wheat and barley[#]

Abstract

Excised leaves were used to eliminate confounding contribution of sodium exclusion mechanisms and evaluate genetic variability in salinity tissue tolerance in a large number of wheat (*Triticum aestivum* and *Triticum turgidum* ssp. *durum*) and barley (*Hordeum vulgare*) accessions. Changes in relative chlorophyll content (measured as chlorophyll content index, CCI) in excised leaves exposed to 50 mM NaCl for 48 h was found to be a reliable indicator of leaf tissue tolerance. In both wheat and barley, relative CCI correlated strongly with overall plant salinity tolerance (evaluated in glasshouse experiments). To a large extent, this tissue tolerance was related to more efficient vacuolar Na^+ sequestration in leaf mesophyll, as revealed by fluorescent Na^+ dye imaging experiments. However, while in barley this correlation was positive, tissue tolerance in wheat correlated negatively with overall salinity tolerance. As a result, more salt-sensitive durum wheat genotypes possessed higher tissue tolerance than bread wheat plants, and this negative correlation was present within each of bread and durum wheat clusters as well. Overall, these results indicate that the lack of effective Na^+ exclusion ability in sensitive wheat varieties is compensated by their better ability to handle Na^+ accumulated in the shoot via tissue-tolerance mechanisms. Implications of these findings for plant breeding for salinity tolerance are discussed.

Keywords

Barley, Chlorophyll content, Excised leaf, Tissue tolerance, Vacuolar Na^+ sequestration, Wheat, *Triticum aestivum*, *Triticum turgidum* ssp. *durum*, *Hordeum vulgare*

Abbreviations

CCI, chlorophyll content index; FV, Na^+ permeable fast-activating tonoplast channels; HKT, high affinity K^+ transporter; MAS, marker assisted selection; NHX, Na^+/H^+ exchanger; ROS, reactive oxygen species; SOS, salt overly sensitive; SV, Na^+ permeable slow-activating tonoplast channels

[#], This chapter has been published as: Wu H, Shabala L, Zhou M, Stefano G, Pandolfi C, Mancuso S, Shabala S (2015) Planta 242: 847−857.

6.1 Introduction

Wheat and barley are amongst the first domesticated species and on a global scale are ranked as the second (549 M mt) and fifth (129 M mt) most important crops in terms of dry matter production (Baik and Ullrich, 2008). Both of them are classified as glycophytes (i.e. species that cannot tolerate long term exposures to saline condition exceeding 200 mM), and their production is strongly affected by soil salinity, with 10% reduction in yield observed at 6.7 and 4.9 dS/m saline water irrigation, respectively (http://www.dpi.nsw.gov.au/agriculture/resources/soils/salinity/crops/tolerance-irrigated). Given the extent of land salinization in the world (Rengasamy, 2006) and the fact that in many countries areas used to cultivate wheat and barley overlap with salinity belt (e.g. in Australia; Colmer et al., 2006), creating salt tolerant wheat and barley germplasm remains one of highest priorities for breeders.

Salinity tolerance is a complex physiological trait composed of numerous sub-traits and controlled by multiple regulatory pathways. This includes (but is not limited to) osmotic adjustment in root and leaf tissues (Munns and Tester, 2008); Na^+ extrusion from root uptake (Maathuis et al., 2014); control of Na^+ loading into the xylem and its retrieval from the shoot (Munns and Tester 2008); vacuolar (Apse et al., 1999; Zhang et al., 2001; Gouiaa et al., 2012) or tissue-specific (Oh et al., 2009; Cuin et al., 2011) Na^+ sequestration; cytosolic K^+ retention (Shabala and Pottosin, 2014); and ROS (reactive oxygen species) detoxification/tolerance (Shabala and Pottosin, 2014).These mechanisms are usually grouped into three major clusters: (i) osmotolerance; (ii) sodium exclusion mechanisms; and (iii) tissue tolerance mechanisms (the latter three traits). Despite the significant progress that has been made in elucidating details of each of these specific mechanisms, the relative contribution of these components to overall salinity tolerance remains unclear, and only a few papers have tried to address this issue in direct experiments (Rajendran et al., 2009; Adem et al., 2014).

Importantly, while each of the tissue tolerance components was characterised in details, both at physiological and molecular levels, little attention was paid to the issue of how these mechanisms are integrated to confer the overall salinity stress tolerance. For example, while vacuole Na^+ sequestration by tonoplast NHX (Na^+/H^+ exchanger) exchangers is considered to be important to confer tissue tolerance in salt-grown plants (Apse et al., 1999; Fukuda et al., 2004b; Munns and Tester, 2008), such sequestration will be not efficient without preventing the back-leak of Na^+ from vacuole via tonoplast slow

(SV) and fast (FV) channels (Bonales-Alatorre et al., 2013a, b) and also without fuelling NHX activity by tonoplast H^+-pumps (Shabala, 2013). Recent studies have added an additional layer of complexity in the relation to vacuolar Na^+ sequestration, reporting low selectivity of NHX between Na^+ and K^+ and assigning the role of NHX antiporters to regulation of K^+ and H^+ homeostasis (Jiang et al., 2010; Bassil et al., 2011; Barragán et al., 2012). Another component of the tissue tolerance is cytosolic K^+ retention (James et al., 2006b; Wu et al., 2013, 2014, 2015d). While a strong correlation between cell's ability to prevent NaCl-induced K^+ efflux from the cytosol in both root and leaf tissues and the overall salinity stress tolerance of various species was reported [e.g. barley (Chen et al., 2005, 2007c; Wu et al., 2013, 2015d), wheat (Cuin et al., 2008; Wu et al., 2013, 2014), lucerne (Smethurst et al., 2008), and poplar (Sun et al., 2009)], the causal relationship between cytosolic K^+ retention and vacuolar Na^+ sequestration remains somewhat elusive. The same is true for ROS detoxification (a third component of tissue tolerance mechanism). While essentiality of keeping ROS levels under control, and an important role of both enzymatic and non-enzymatic antioxidant (AO) systems in plant salt stress responses are beyond any doubt (Ahmad et al., 2010; Gill and Tuteja, 2010), much less is known of how changing cytosolic K^+ and Na^+ concentrations affects cell's AO activity.

The use of a whole-plant phenotyping, while proved to be efficient in separating various tolerance components (James et al., 2002; Munns and James, 2003; Rajendran et al., 2009), has a potential caveat of ignoring genotypic variability in plant's ability to control Na^+ delivery to the shoot. Indeed, the lack of visual symptoms of salt stress such as chloropsis or necrosis in plant leaves after a month of growing under saline irrigation may be attributed to either efficient vacuolar Na^+ sequestration in leaf mesophyll cells (hence, higher tissue tolerance), or higher SOS (salt overly sensitive) -like activity in root epidermis (hence, better Na^+ extrusion from uptake (Cuin et al., 2011), or higher rate of Na^+ retrieval from the xylem (Munns et al., 2012).

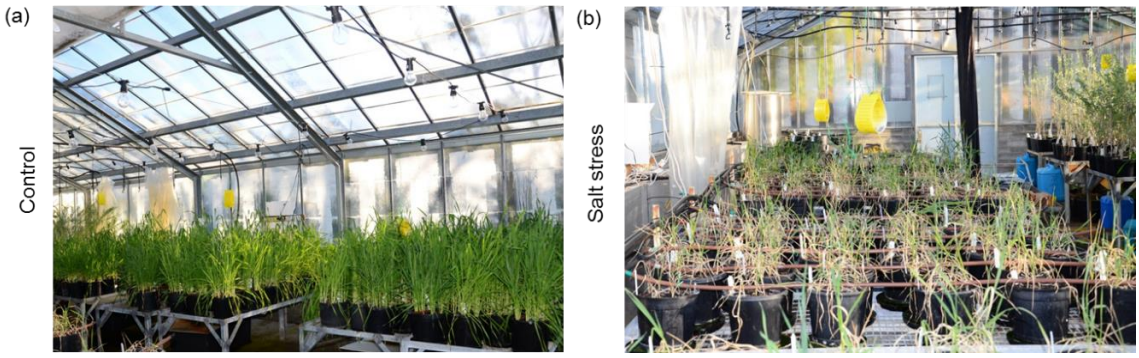
The above problem may be partially resolved if excised leaves are used instead of whole plants, so the same amounts of Na^+ will be present in the xylem sap within leaves at each specific time. Earlier we have validated this method by comparing the difference in tissue tolerance between leaves of four barley varieties contrasting in salinity stress tolerance (Shabala et al., 2010). In the current work, we extend that preliminary work to a broad range of barley and wheat accessions by screening tissue tolerance component of nearly 50 barley and 50 wheat genotypes with different overall salinity tolerance.

Being glycophyte species, barley is nonetheless classified as "salt includer" (Perez-Lopez et al., 2010; Mian et al., 2011) and possess strong vacuolar Na^+ sequestration ability (Garthwaite et al., 2005; James et al., 2006b). On the contrary, wheat is regarded as "salt excluder" (Kingsbury et al., 1984; Cuin et al., 2008; He et al., 2010) and varieties with superior tolerance show higher shoot Na^+ exclusion ability (Colmer et al., 2005; Garthwaite et al., 2005). Thus, another aim of this work was to compare the role of the tissue tolerance component between wheat and barley, two cereal species with contrasting strategies of dealing with salinity.

6.2 Materials and methods

6.2.1 Plant materials and growth conditions

Forty-seven barley (*Hordeum vulgare* L.) genotypes and forty-five wheat genotypes (25 bread wheat, *Triticum aestivum*; and 20 durum wheat, *Triticum turgidum* spp. *durum*) contrasting in their salinity tolerance were used in this study. All wheat seeds were received from the Australian Winter Cereals Collection while barley seeds were obtained from multiple sources and multiplied in our laboratory. Plants were grown between February and December 2013 in the glasshouse facilities at the University of Tasmania essentially described in Chen et al. (2007d). Plants were sown in 4.5 L PVC pots filled with a standard fertilised potting mix (see (Chen et al. 2007d) for details). A saucer was placed under each pot, and the plants were irrigated three times per day by an automatic watering system (Online Resource 1).

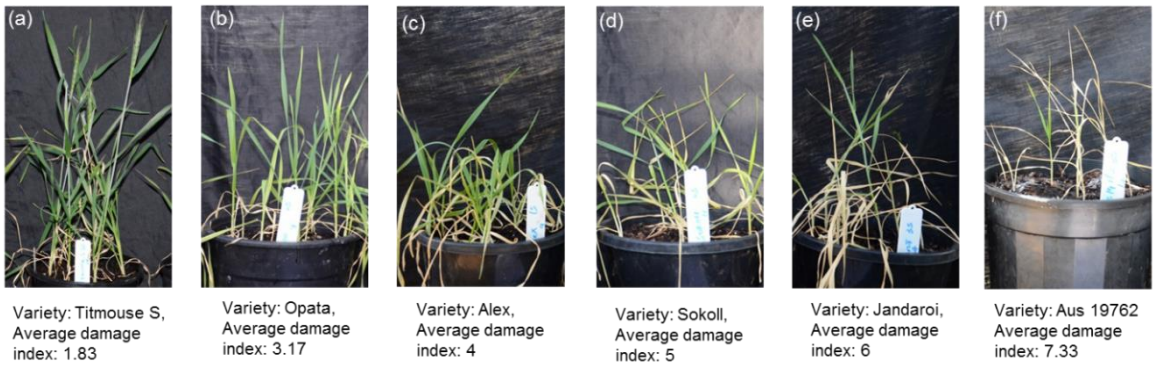


Online Resource 1. Genetic variability of overall salt tolerance in wheat grown in glasshouse under 300 mM NaCl for 5 weeks. **a** Plants were irrigated with tap water (control). **b** Salt treatment (plants irrigated with 300 mM NaCl).

6.2.2 Glasshouse experiments and overall salt tolerance evaluation

Twelve to 14 seeds of each variety were sown in 4.5 L PVC pots filled with the standard potting mix, in triplicates. All varieties were labelled by serial numbers, and

arranged randomly in the glasshouse. After emergence plants were thinned to leave eight uniform seedlings in each pot, and salt treatment (300 mM NaCl) was applied for five weeks. No apparent symptoms of leaf chlorosis were found in wheat grown under control conditions which is irrigated with tap water (Online Resource 1a) but five weeks of salinity treatment resulted in a clear differentiation between varieties (Online Resource 1b). The visual symptoms of salinity damage were scored as the damage index on zero to 10 scale (0 – no visual symptoms of damage; 10 – plants are dead) using criteria such as the extent of leaf chlorosis, numbers of dead leaves, and plant survival rate (Zhou et al., 2011; Wu et al., 2014; Online Resource 2). Each data point of the salt stress induced damage index was an average from 8 seedlings in a pot. The final value of the salt stress-induced damage index for each variety was the averaged results from three replicates (three different pots for each variety). The above scoring was conducted as a blind test to avoid any subjectivity. The aboveground biomass was then harvested, and samples were dried at 65 °C for 72 h in a Unitherm Dryer (Birmingham, UK) for further analyses.

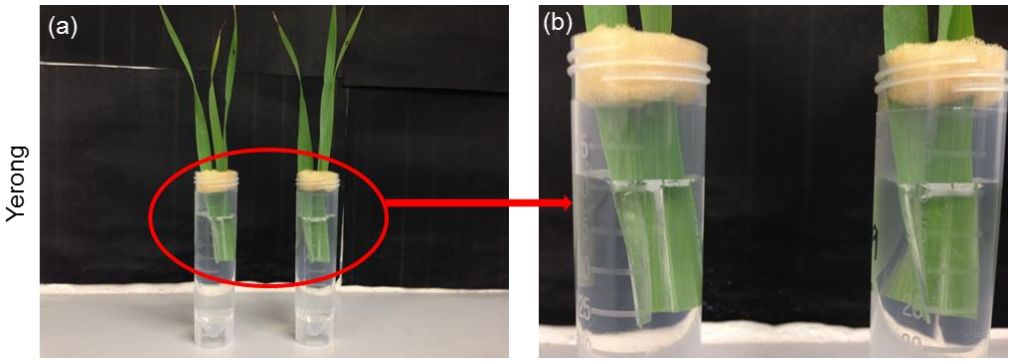


Online Resource 2. Quantification of impact of salt stress on wheat performance by scoring of the damage index (0 to 10 scale). 0 – no visual symptoms of damage; 10 –dead plants. Plants were grown in glasshouse and treated with 300 mM NaCl for 5 weeks. The genetic variability in salt stress susceptibility and scoring index is illustrated by six representative varieties: a Titmouse S (average scoring index 1.83). b Opata (average scoring index 3.17). c Alex (average scoring index 4). d Sokoll (average scoring index 5). e Jandaroi (average scoring index 6). f Aus 19762 (average scoring index 7.33).

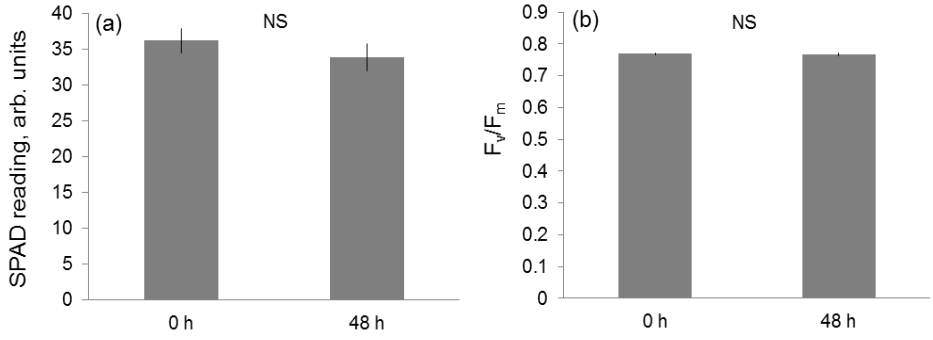
6.2.3 Experiments with excised leaves

An experimental protocol to rapidly screen plants tissue tolerance to salinity was developed. In a separate set of experiments, plants were grown under control conditions (irrigated with tap water) as described above until five weeks old. The third youngest fully expanded leaf was excised at the leaf base and immediately inserted into a container containing tap water. Excised leaves were brought into the laboratory, further cut under the running tap water (to avoid a possible embolism) to the uniform size, and then inserted into a sealed 50 mL Falcon tubes containing 1/8 Hoagland solution with or

without 50 mM NaCl (usually the Scholander measured Na^+ concentration in the xylem steam of salt stressed plants). The choice of this concentration was driven by the fact that 20 to 50 mM apoplastic Na^+ concentrations are typically found in the xylem sap of plants grown under saline conditions (e.g. Shabala, 2013; Bose et al., 2014b). Thus, our experimental conditions mimicked ones expected to be found in a field. Three individual leaves were inserted into each tube and immobilised by an elastic sponge (Online Resource 3). All leaves were immersed in 40 mL of solution by the same depth (~ 30 mm), and tubes were sat on the steel racks in a growth cabinet ensuring the homogeneous light conditions for each leaf. The intensity of light in the growth cabinet was 150 µmol m^{-2} s^{-1} irradiance; 14h/10h day/night cycle and 26/20 ± 1.0 °C day/night temperatures. No significant chlorophyll content change (estimated by SPAD reading) and impairment of photosynthesis capacity (estimated by F_v/F_m value) were observed in excised leaves under control condition after 48h (Online Resource 4). The experiment was repeated in duplicate (with 6 biological replicates).



Online Resource 3. Plant screening for salinity tissue tolerance in leaves. Excised barley leaves (variety Yerong) were placed in 50 mL Falcon tubes sealed with sponge at the top (a), with the leaf cut edge immersed into appropriate solution by the same depth (~30 mm) (b).



Online Resource 4. Chlorophyll content (**a**) and maximum photochemical efficiency of PSII (F_v/F_m chlorophyll fluorescence values) (**b**) in excised barley leaves (variety Yerong) under control condition at the beginning (time zero) and at the end (48 h) of the experiment. No statistically significant (at $P < 0.05$) difference in either characteristic was observed. NS = not significant at $P < 0.05$. Mean ± SE, $n = 6$.

Leaf chlorophyll content index (CCI) was measured by a chlorophyll meter (SPAD-502, Konica Minolta, Japan) at time zero (immediately after excision) and in 48 h. The CCI values of salt stressed excised leaves were measured from the top part of the leaf blade since this area shows the most severe symptom of chlorosis. The sample size was 20 to 30 replicates, sufficient to account for any technical error. The maximal photochemical efficiency of photosystem II (chlorophyll fluorescent F_v/F_m value) was quantified by a chlorophyll fluorometer (OS-30p, Opti-sciences, Hudson, NH, USA) at the same time. For measuring the Fv/Fm, samples were kept under darkness for 15 min to remove all the electrons from the electron transport chain and make all photosynthetic reaction centres fully oxidized. Leaves were then placed into 1.5 mL Eppendorf tubes and stored in a freezer at -20 °C for sap nutrient analysis. A transpiration rate was calculated by the difference between the measuring amounts of water evaporated from each tube. For transpiration measurement, the amount of evaporated water was measured by quantifying amount of remaining water.

6.2.4 Leaf ion content

Sap Na^+ content in salt-stressed excised leaves was analysed from freeze-thawed leaf samples as described elsewhere (Cuin et al., 2009). Dry leaf samples from the salt-stressed whole plants in the glasshouse experiment were digested in a 120 mL Teflon digestion vessel in a microwave digester (MDS-2000 microwave digestion system, CEM Corporation, USA) using 70% HNO3 (5 mL) and 30% H_2O_2 (2 mL) per 0.1 g specimen. Both sap and digested samples were centrifuged at 5, 000 *g* for 10 min at room temperature (Avanti J-30I centrifuge, Beckman Coulter, Germany). After centrifuging, 10 µL of the extracted sap or 0.2 mL of the digested solution was diluted to a final volume of 10 mL in 25 mL beakers, and Na^+ content was measured by a Flame Photometer (PFP7, Jenway, UK).

6.2.5 Laser confocal microscopy measurements

Barley plants were grown and excised leaves exposed to salinity as described above. Leaf segments of about 5 x 5 mm were then cut from the middle part of the leaf, avoiding major veins. Eight to 10 segments were incubated in Eppendorf tubes in 500 µL of the 10 µM Sodium Green solution (S-6901, Molecular Probes). After 1 h of incubation in darkness, the samples were examined using confocal microscopy as described in our previous publication (Cuin et al., 2011). All specific details for methodological aspects

related to the use of fluorescent Na^+ dye and quantification of intracellular Na^+ distribution between the vacuole and cytosol are available from Wu et al. (2015a) and Bonales-Alatorre et al. (2013b). In brief, dye was reconstituted as a stock with anhydrous dimethyl sulfoxide before use. Confocal imaging was performed using an upright Leica Laser Scanning Confocal Microscope SP5 (Leica Microsystems, Heidelberg, Germany) equipped with a 40× oil immersion objective. The excitation wavelength was set at 488 nm, and the emission was detected at 510-520 nm. Images were analysed with LAS AF software (Leica Microsystems). Cytosolic (chloroplast) and vacuolar signals were measured as mean intensity of the representative area of the same size within appropriate compartment. The background signal was measured from the empty region of the similar size, and then subtracted from the measured signal. Comparison of different levels of fluorescence between cells was carried out by visualizing cells with the identical imaging settings of the confocal microscope (i.e. exposure times, laser intensity, pinhole diameter and settings of the imaging detectors).

6.2.6 Statistical analysis

All data (given as mean ± SE, n = sample size) were analysed by using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA). All of the replicates are biological replicates. Comparison of different parameters between tolerant and sensitive groups was done using independent samples t-test. Asterisks indicate significance levels at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively. The significance of correlations between different parameters was determined by Bivariate Correlations based on two-tailed Pearson Correlation. The data used for correlation analysis are the average values of measured independent parameters for each variety.

6.3 Results

6.3.1 Methodological aspects of plant screening for tissue tolerance

Two barley varieties (TX 9425, tolerant; and Naso Nijo, sensitive; Fan et al., 2014) contrasting in their salt tolerance were used for quantifying the leaf tissue tolerance in preliminary experiments. After 48h treatment, salt-treated (50 mM NaCl) leaves showed a clear sign of chlorosis (Fig. 1b), which was reflected in a significant decline in the relative chlorophyll content (CCI values expressed as % of control; Fig 1c). This decline was much more severe in the salt-sensitive Naso Nijo genotype compared with tolerant TX9425 (significant at $P < 0.01$). At the same time, no significant (at $P < 0.05$)

difference in the transpiration rate between these varieties was found (Fig. 1d). Thus, despite the amount of Na^+ brought into the leaf mesophyll by transpiration stream was the same in both the varieties, the extent of the damage to photosynthetic machinery (judged by the relative chlorophyll content) was strikingly different.

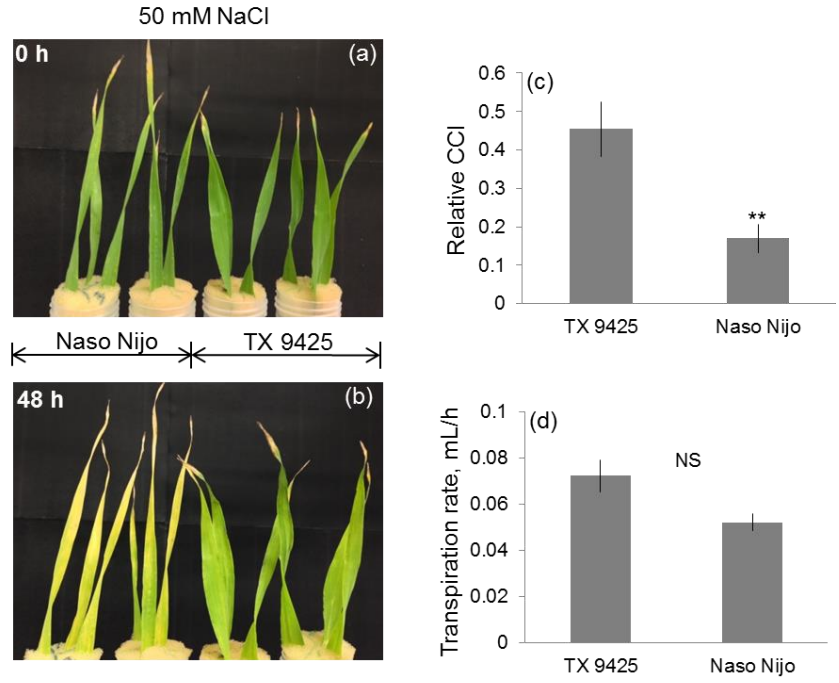


Fig. 1. Chlorophyll content and transpiration parameters in excised barley leaves exposed to 50 mM salinity treatment for 48 h. Two contrasting varieties - TX 9425 (salt tolerant) and Naso Nijo (salt sensitive) - were used. **a**, **b** Leaf appearance before (time zero) and 48 hours after salinity exposure. **c** Relative chlorophyll content (CCI; % of control) **significant at $P < 0.01$. **d** Transpiration rate. NS = not significant at $P < 0.05$. Mean ± SE, $n = 6 – 9$.

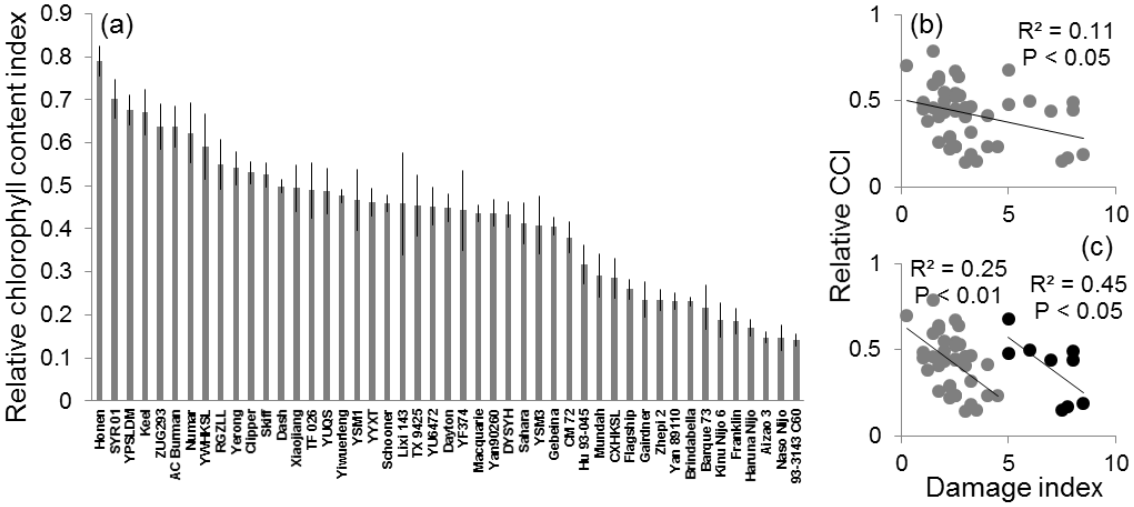


Fig. 2. Genetic variability in relative CCI in salt stressed excised barley leaves. **a** Relative (% of control) CCI values of all 47 barely varieties measured after 48 h exposure to 50 mM NaCl. **b** Correlation between relative CCI and overall salinity tolerance in barley (estimated as salt stress-induced damage index scored in glasshouse experiments; higher numbers denote susceptible varieties). **c** Correlation between relative CCI and damage index (scored in glasshouse experiments) amongst 47 barley varieties grouped into two clusters: tolerant (with the damage index < 5), and sensitive (with the damage index > 5). Mean ± SE, $n = 6 – 9$. Each point represents a variety.

6.3.2 Barley tissue tolerance correlates with the overall salinity stress tolerance

To test whether tissue tolerance contributes to the overall salinity tolerance in barley, 47 barley varieties were screened. The relative CCI in barley ranged from the lowest 0.14 ± 0.01 (in cultivar 93-3143 C60) to the highest 0.79 ± 0.03 (in cultivar Honen) (Fig. 2a). A significant positive correlation (r = 0.33, $P < 0.05$; Fig. 2b) between the relative CCI and the overall salinity tolerance (judged by a damage index at harvest; Online Resource 5) was found. When plants were divided into two groups: tolerant (with damage index < 5), and sensitive (with damage index > 5), this correlation became much stronger within each group assessed (r = 0.50 and 0.67, respectively) (Fig 2c).

6.3.3 Tissue tolerance in barley is related to higher vacuolar Na^+ sequestration ability in the leaf mesophyll

Bread wheat		Durum wheat		Barley			
Variety	Index	Variety	Index	Variety	Index	Variety	Index
Berkut	1.83	Alex	4	SYR01	0.25	AC Burman	2.67
Kharchia 65	1.83	Zulu	5.33	TX	1	Clipper	2.75
Titmouse S	1.83	Citr 7792	5.67	YUQS	1	Schonner	3
Cranbook	2.5	AUS12746	5.83	CM72	1.25	93-3143 C60	3
Excalibur	2.5	Covelle	5.83	Honen	1.5	Lixi 143	3
Drysdale	2.83	Kalka	6	YWHKSL	1.5	YSM3	3
Westonia	3	Jandaroi	6	YYXT	1.5	Franklin	3.25
Opata	3.17	Tehuacan 60	6.17	Flagship	1.75	Hu 93-045	3.25
H7747	3.17	AUS16469	6.33	Gebeina	1.75	YSM1	3.25
Persia 6	3.17	Biskri ac2	6.33	Numar	1.75	Aizao 3	3.5
Persia 21	3.33	Purple Grain	6.33	ZUG293	1.75	Sahara	4
India 38	3.33	Tiimilia	6.33	DYSYH	2	Gairdner	4
Kukri	3.5	Hyperno	6.5	RGZLL	2	Yan89110	4.5
Halberd	3.83	Tamaroi	6.5	Xiaojiang	2	Yiwu Erleng	5
Sevillie 20	3.83	Jori	6.5	YU6472	2	YPSLDM	5
Iran 118	4.17	AUS19762	7.33	Barque 73	2.25	Dash	6
Iraq 43	4.17	Caparoi	7.5	CXHKSL	2.25	Macquarie	7
Iraq 50	4.17	Towner	7.83	Mundah	2.25	NN	7.5
Belgrade 3	4.33	C250	7.83	Keel	2.5	Haruna Nijo	7.75
Krichuaff	4.5	Citr 7805	8.16	Skiff	2.5	TF026	8
Sokoll	5			Dayton	2.5	YF374	8
Frame	5.17			Yan90260	2.5	KINU NIJO 6	8.5
Janz	5.17			Yerong	2.5		
340	5.5			Zhepi 2	2.5		
Baart 46	5.83			Brindabella	2.5		

Online resource 5. Glasshouse experiments of 25 bread wheat, 20 durum wheat, and 47 barley varieties under long term salt stress (300 mM NaCl, ~5 weeks). Damage index was scored based on the extent of chlorosis and plant survival rate on zero to 10 scale (0 – no visual symptoms; 10 – dead plants).

Fluorescence dye microscopy was used to quantify Na^+ distribution within the leaf mesophyll cells using another set of contrasting barley cultivars (Numar, tolerant; and Gairdner, sensitive; Chen et al., 2007c; Fig. 3a, b). In salt-tolerant cultivar Numar, most of Na^+ was localised in the mesophyll cell's vacuole, and the fluorescent signal from this compartment exceeded the one from chloroplasts by 2-fold (54.9 ± 4.9 vs 28.5 ± 1.2; Fig 3c). In a contrast, in a salt-sensitive cultivar Gairdner the chloroplast signal was 4-fold higher compared with that originating from the vacuole (49.6 ± 3.1 vs 11.8 ± 0.8; Fig. 3d), indicating poor vacuole Na^+ sequestration ability in the latter variety.

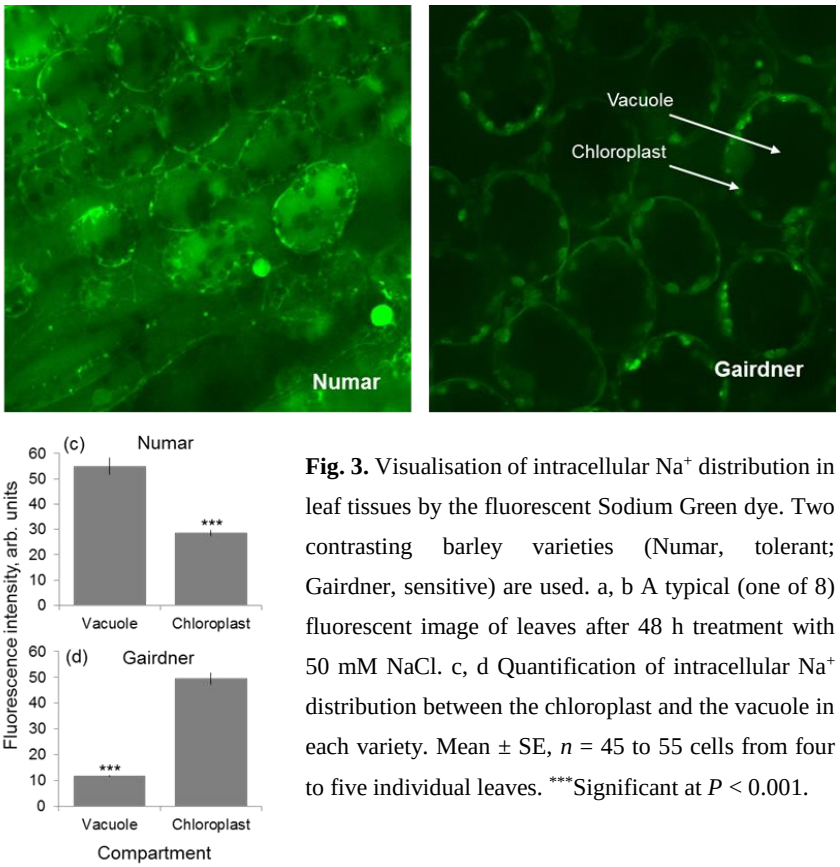


Fig. 3. Visualisation of intracellular Na^+ distribution in leaf tissues by the fluorescent Sodium Green dye. Two contrasting barley varieties (Numar, tolerant; Gairdner, sensitive) are used. a, b A typical (one of 8) fluorescent image of leaves after 48 h treatment with 50 mM NaCl. c, d Quantification of intracellular Na^+ distribution between the chloroplast and the vacuole in each variety. Mean ± SE, n = 45 to 55 cells from four to five individual leaves. ***Significant at $P < 0.001$.

6.3.4 Durum wheat possesses higher tissue tolerance compared with bread wheat

Two contrasting bread wheat varieties (Kharchia 65, tolerant; and Baart 46, sensitive; Cuin et al., 2009) were used in preliminary experiments. Leaf exposure to 50 mM NaCl treatment for 48 h has resulted in a significant decline in chlorophyll content (Fig. 4). Pronounced chlorosis symptoms were observed in both genotypes (Fig. 4 a, b). Similar to barley, no significant (at $P < 0.05$) difference in transpiration rate was found between the genotypes (Fig. 4d). However, to a great surprise the negative impact of salinity on CCI was much more pronounced in (overall) salt-tolerant Kharchia 65 genotype (relative CCI values 0.26 ± 0.05 compared with 0.52 ± 0.07 for a sensitive Baart 46; Fig. 4c).

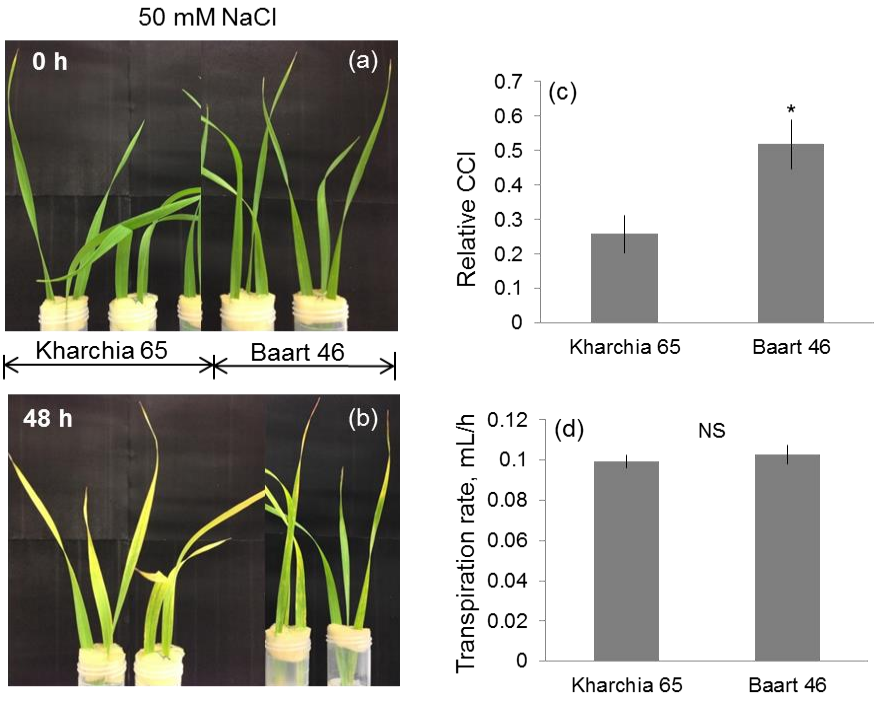


Fig. 4 Chlorophyll content and transpiration parameters in excised wheat leaves exposed to 50 mM NaCl for 48 h. Two contrasting varieties – Kharchia 65 (salt tolerant) and Baart 46 (salt sensitive) - were used. **a**, **b** Leaf appearance before (at time zero) and 48 hours after salinity treatment. **c** Relative chlorophyll content (CCI; % to control) **significant at $P < 0.01$. **d** Transpiration rate. NS = not significant at $P < 0.05$. Mean ± SE, $n = 6 - 9$.

To follow this counterintuitive observation, 45 bread and durum wheat varieties were screened, to investigate the relationship between tissue tolerance and the overall salinity tolerance in these species. Strong (nearly 15 folds) variability of relative chlorophyll content (CCI) was found (Fig. 5a). In bread wheat, Persia 6 showed the highest relative CCI (0.57 ± 0.08) and India 38 showed the lowest one (0.05 ± 0.01) (Fig. 5a). In durum wheat, the highest relative CCI was found in Aus 16469 (0.67 ± 0.03) and the lowest was in Alex (0.16 ± 0.06) (Fig. 5a). Consistent with our previous observations reported in Fig 4, negative impact of apoplastic NaCl on leaf CCI values was stronger in bread (0.25 ± 0.02) than in durum wheat (0.45 ± 0.03) (Fig. 5b; significant at $P < 0.001$). Consistent with the comparison of just two contrasting wheat varieties (Fig. 4) and in a contrast with the data reported for barley (Fig. 2b, c), relative CCI and the overall salinity tolerance (measured as a damage index at harvest; Online Resource 5) in wheat were correlated negatively ($r = 0.77$, $P < 0.001$; Fig. 5c). The negative correlation was found in both bread ($r = 0.48$, $P < 0.05$; Fig. 5d) and durum ($r = 0.69$, $P < 0.001$; Fig. 5e) wheat clusters.

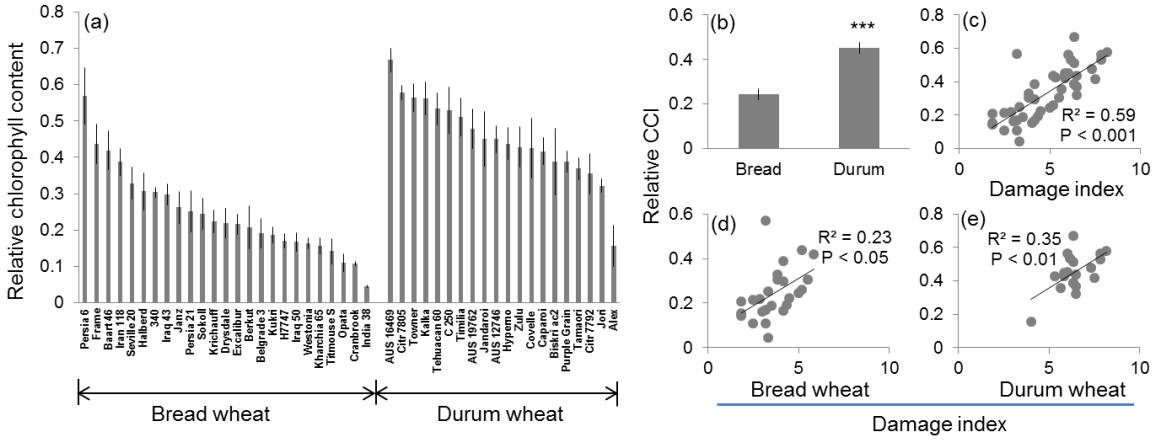


Fig. 5. Genetic variability in the relative CCI in salt stressed excised wheat leaves. **a** Relative (% of control) CCI values of durum and bread wheat varieties measured after 48 h exposure to 50 mM NaCl. Mean ± SE, *n* = 6. **b** Mean relative CCI values for durum and bread wheat pools. Mean ± SE, *n* = 20 and 25 varieties, respectively. ***Significant at $P < 0.001$. **c** Correlation between relative CCI and salinity damage index (scored in glasshouse experiments) in all 45 wheat varieties screened. **d**, **e** Correlation between relative CCI and overall salinity tolerance within durum and bread wheat clusters. Each point represents a variety.

The above difference in the extent of NaCl damage to leaf photosynthetic machinery was not related to a higher rate of delivery of salt into leaf mesophyll in bread wheat, as no significant (at $P < 0.05$) difference of transpiration rate was found between the pooled values of bread and durum wheat (Fig. 6b). Similarly, no significant correlation between relative CCI and transpiration rate was found in wheat (r = 0.28, $P > 0.05$; Fig. 6c).

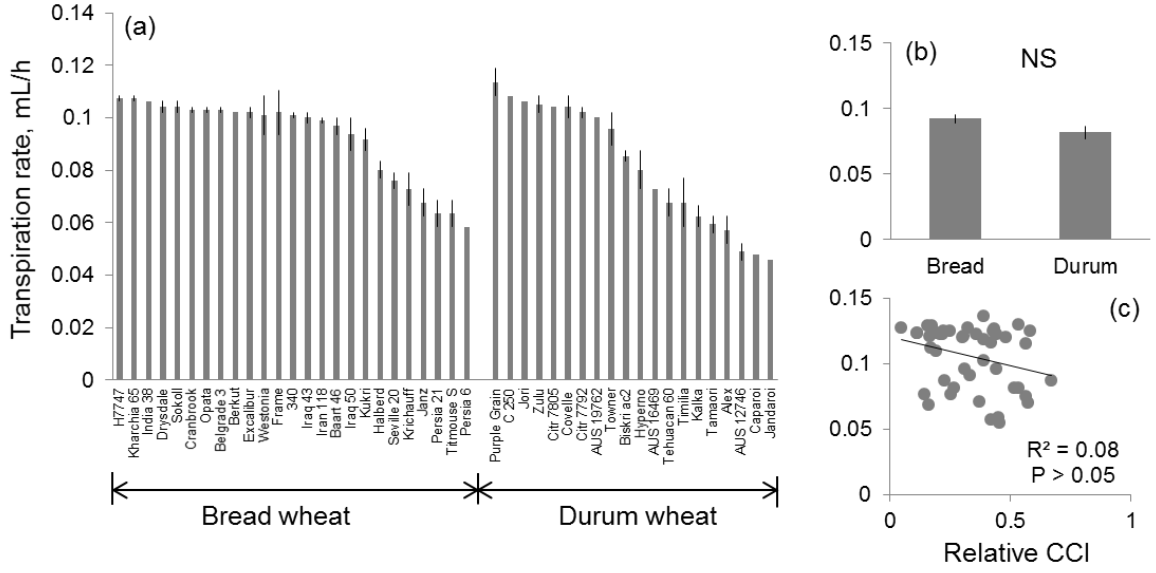


Fig. 6. Genetic variability in transpiration rate in wheat. **a** Transpiration rate values among 45 durum and bread wheat varieties. Mean ± SE, *n* = 6. **b** Pooled transpiration rate for bread and durum wheat clusters. NS = not significant at $P < 0.05$. Mean ± SE, *n* = 25 and 20 varieties, respectively. **c** Correlation between transpiration rate and relative CCI in all 45 wheat varieties screened. Each point represents a variety.

6.3.5 Patterns of Na^+ accumulation in wheat leaves

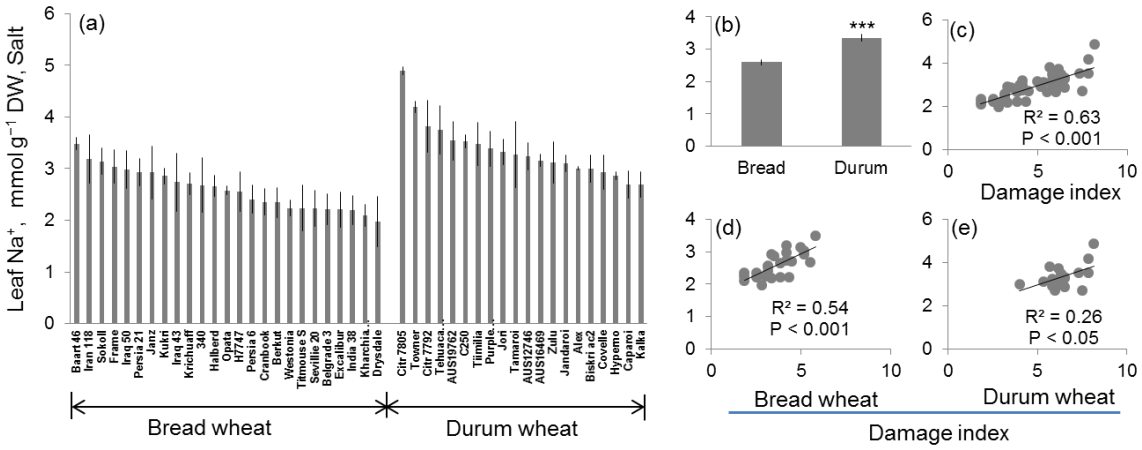


Fig. 7. a Leaf Na^+ content in wheat grown under saline conditions (300 mM NaCl irrigation for 5 weeks). **b** Pooled leaf Na^+ values within durum and bread wheat clusters. Mean ± SE, n= 20 and 25 varieties, respectively. **c** Correlation between leaf Na^+ content and salt stress induced damage index (scored in glasshouse experiments) in 45 wheat varieties. **d**, **e** Correlation between leaf Na^+ content and salt stress-induced damage index (scored in glasshouse experiments) analysed separately within durum and bread wheat clusters. Each point represents a variety.

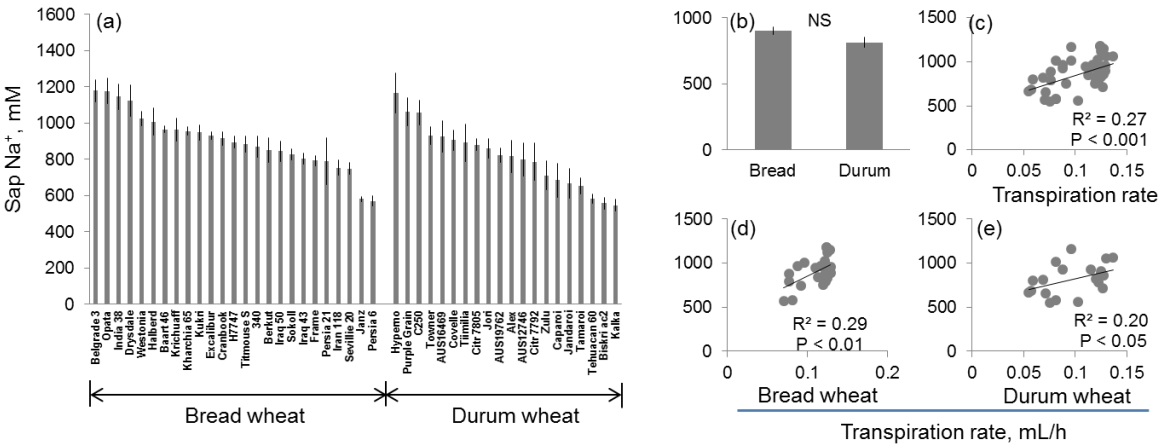


Fig. 8. a Leaf sap Na^+ concentration in excised wheat leaves exposed to 50 mM NaCl for 48 h. Mean ± SE, $n = 6$. **b** Pooled leaf sap Na^+ concentrations for bread and durum wheat clusters. NS = not significant at $P < 0.05$. Mean ± SE, $n = 25$ and 20 varieties, respectively. **c** Correlation between sap Na^+ content in excised leaves and transpiration rate in all 45 wheat varieties screened. **d**, **e** Correlation between sap Na^+ content in excised leaves and transpiration rate analysed separately within durum and bread wheat clusters. Each point represents a variety.

Leaf Na^+ contents in soil-grown plants treated with 300 mM NaCl for 5 weeks were substantially higher in durum compared with bread wheat (Fig. 7 a) and correlated positively with the overall salt induced damage index scored in glasshouse experiments ($r = 0.79$, $P < 0.001$; Fig. 7c). The same trend was observed for each of the clusters: bread ($r = 0.73$, $P < 0.001$; Fig. 7d) and durum wheat ($r = 0.51$, $P < 0.05$; Fig. 7e). Overall, pooled leaf Na^+ content in durum wheat exceeded that of bread wheat by roughly 30% (3.35 ± 0.12 vs 2.60 ± 0.08 mmol g^{-1} DW, respectively; significant at $P < 0.001$; Fig. 7b).

However, when 50 mM NaCl was added directly to the transpiration flow (in experiments with excised leaves), no significant differences between the sap Na^+ contents in salt-stressed excised leaves in bread and durum wheat were found (Fig. 8a, b). A significant and positive correlation between transpiration rate and sap Na^+ content in salt stress excised leaves was found in wheat ($r = 0.52$, $P < 0.001$; Fig. 8c). Similar results were also found separately for both bread ($r = 0.54$, $P < 0.01$; Fig. 8d) and durum ($r = 0.45$, $P < 0.05$; Fig. 8e) wheat clusters

6.4 Discussion

6.4.1 Tissue tolerance in barley

Barley is classified as the most salt-tolerant species of the cereals (Munns and Tester, 2008), and it was suggested that this tolerance is most likely achieved by effective Na^+ compartmentalization in leaf vacuoles (Garthwaite et al., 2005). Here we provide a direct experimental support for this notion. A significant positive correlation was observed between relative CCI and the overall salinity tolerance in each of barley groups (tolerant and sensitive; Fig. 2c) suggesting an important role of the tissue tolerance in the overall salinity stress tolerance in this species. Using fluorescent Na^+ dye we also showed that at least a part of this tissue tolerance is conferred by more efficient vacuolar Na^+ sequestration in tolerant varieties (Fig. 3).

Given the complexity of the salinity tissue tolerance mechanisms, the higher tissue tolerance in salt-tolerant barley genotypes reported in this work (Fig. 2) cannot be solely attributed to any single mechanism. While vacuolar Na^+ sequestration ability is usually considered as a main component of tissue tolerance (Munns and Tester, 2008), it cannot be attributed to merely higher activity of tonoplast NHX Na^+/H^+ exchangers, as originally believed (Apse et al., 1999). At a very least, all the Na^+ pumped into vacuole has to be safely locked in there and prevented from the back-leak into cytosol (Pantoja et al., 1989; Glenn et al., 1999; Shabala, 2013). This implies an efficient control over permeability of tonoplast slow (SV) and fast (FV) vacuolar channels (Bonales-Alatorre et al., 2013a, b), and it was shown recently that stress-induced increase in the level of compatible solutes (specifically, choline) may be pivotal to this process (Pottosin et al., 2014a). Also, we have shown earlier that barley varieties used in this work differ dramatically in the ability to retain K^+ in leaf mesophyll under saline conditions (Wu et al., 2015d), with strong positive correlation reported between this trait and the overall salinity tolerance. At the

same time, high cytosolic K^+ content is essential for a normal operation of tonoplast H^+-PPase (Serrano et al., 2007) that is essential to "fuel" Na^+/H^+ exchanger.

The observed strong localisation of fluorescent Na^+ signal to chloroplasts in salt-sensitive barley variety (Fig. 3) may also be an important contributing factor. Stress-induced increase in chloroplast Na^+ content was previously reported in a range of plant species (Robinson et al., 1984; Wang et al., 2007; Slabu et al., 2009) and was found to disturb metabolism and finally impair the photosynthetic activity (Yeo and Flowers, 1986). The accumulation of excessive Na^+ in chloroplasts may directly cause ion toxicity and induce the subsequent oxidative stress (Hernández et al., 1995, Savouré et al., 1999), and may exhibit direct and indirect restrictions on dark and light reactions (Wang et al., 2007). It remains to be answered of whether higher chloroplast Na^+ accumulation in sensitive varieties was attributed to poor ability of chloroplast transport systems to prevent Na^+ from entering stroma, or higher vacuolar sequestration ability in tolerant varieties was sufficient to keep chloroplasts "sodium free".

6.4.2 Tissue tolerance in wheat

To our great surprise, a strong negative correlation between tissue tolerance and overall salinity tolerance was found in either bread or durum wheat (Fig. 5), and tissue tolerance in durum wheat well exceeded one in bread wheat (Fig. 5). We interpret this as evidence for a compensation mechanism aimed to protect varieties with poor Na^+ exclusion ability.

As shown in Fig. 7, leaf Na^+ content correlated negatively with the overall salinity tolerance in soil-grown plants, and was significant lower in bread than durum wheat (Fig. 7b). This is consistent with the general concept that bread wheat has better Na^+ exclusion ability (Gorham, 1987; Shah et al., 1987; Cuin et al., 2008) and possess stronger overall salinity tolerance (Cuin et al., 2008). This Na^+ extrusion ability may be due to higher rate of Na^+ efflux into rizhosphere origination from higher SOS1 Na^+/H^+ exchanger activity at root plasma membrane (Cuin et al., 2011), better control of Na^+ loading into the xylem (Tester and Davenport, 2003), or higher rate of Na^+ retrieval back into roots by HKT transporters at xylem parenchyma interface (Sunarpi et al., 2005; Horie et al., 2009). Once these mechanisms have been bypassed by adding Na^+ directly to leaf apoplast (using excised leaves, as in this work), no significant difference in leaf Na^+ content between bread and durum wheat was found (Fig. 8). Yet, leaf tissue tolerance in durum wheat exceeded that in bread wheat by nearly 2-fold (Fig. 5b). It is plausible then to suggest that the lack of effective Na^+ exclusion ability in sensitive varieties is

compensated by their better ability to handle Na^+ accumulated in the shoot via tissue-tolerance mechanisms. In this context, it is worth mentioning that earlier Gregorio et al. (2002) reported that tolerant rice lines also showed the lack of tissue tolerance ability.

6.4.3 Implications for breeders

Despite some recent successes (e.g. Munns et al., 2012), the progress in plant breeding for salinity stress tolerance is slower when one may wish. While performing better than non-transformed controls, transgenic wheat plants overexpressing *HKT* (high affinity K^+ transporter) (James et al., 2002; Munns et al., 2012) or *NHX1* (Xue et al., 2004) genes still show significant (up to 50%) yield penalties compared with non-saline plants. It was argued that some of the traits the breeders tried to targets were often not compatible, and that salinity stress tolerance may be achieved only via pyramiding approach (Flowers and Yeo, 1995, Shabala, 2013). In this case, targeting vacuolar Na^+ sequestration to improve the leaf tissue tolerance and pyramiding it with other salt tolerance mechanisms might be beneficial to the superior salt tolerant barley breeding program.

Furthermore, our results reported here also highlight the possible caveats of the whole-plant phenotyping as a breeding strategy. Indeed, if one is after genes conferring tissue tolerance in wheat then salt-*sensitive* varieties should be used as gene donors for this trait. This recommendation is counter-intuitive and, until now, was not taken into account by breeders. The suggested method of using excised leaves to overcome the confounding effects of Na^+ exclusion mechanisms on quantification of tissue tolerance trait may overcome this limitation and arm breeders with effective way of screening plants for this important trait. This will allow to better use available Marker Assisted Selection (MAS) tools to create/breed a robust wheat and barley varieties which can perform well in saline soils.

Chapter 7

Linking salinity stress tolerance with tissue-specific Na^+ sequestration in wheat roots[#]

Abstract

Salinity stress tolerance is a physiologically complex trait that is conferred by the large array of interacting mechanisms. Among these, vacuolar Na^+ sequestration has always been considered as one of the key components differentiating between sensitive and tolerant species and genotypes. However, vacuolar Na^+ sequestration has been rarely considered in the context of the tissue-specific expression and regulation of appropriate transporters contributing to Na^+ removal from the cytosol. In this work, six bread wheat varieties contrasting in their salinity tolerance (three tolerant and three sensitive) were used to understand the essentiality of vacuolar Na^+ sequestration between functionally different root tissues, and link it with the overall salinity stress tolerance in this species. Roots of 4-d old wheat seedlings were treated with 100 mM NaCl for 3 days, and then Na^+ distribution between cytosol and vacuole was quantified by CoroNa Green fluorescent dye imaging. Our major observations were as follows: 1) salinity stress tolerance correlated positively with vacuolar Na^+ sequestration ability in the mature root zone but not in the root apex; 2) Contrary to expectations, cytosolic Na^+ levels in root meristem were significantly higher in salt tolerant than sensitive group, while vacuolar Na^+ levels showed an opposite trend. These results are interpreted as meristem cells playing a role of the "salt sensor"; 3) No significant difference in the vacuolar Na^+ sequestration ability was found between sensitive and tolerant group in either transition or elongation zones; 4) The overall Na^+ accumulation was highest in the elongation zone, suggesting its role in osmotic adjustment and turgor maintenance required to drive root expansion growth. Overall, the reported results suggest high tissue-specificity of Na^+ uptake, signalling, and sequestration in wheat root. The implications of these findings for plant breeding for salinity stress tolerance are discussed.

Keywords

Bread wheat, cytosolic Na^+, Na^+ distribution, root zones, salinity stress tolerance, vacuolar Na^+ sequestration

[#], This chapter has been published as: Wu H, Shabala L, Liu X, Azzarello E, Zhou M, Pandolfi C, Chen ZH, Bose J, Mancuso S, Shabala S (2015) Frontiers in Plant Science 6: 71.

7.1 Introduction

More than 800 million hectares (6%) of land are affected by salinity worldwide (Munns and Tester, 2008). As sodium is one of the most abundant metal elements, sodium salts dominate in many saline soils of the world (Rengasamy, 2006). High concentrations of salts in soils account for large decreases in the yields of a wide variety of crops all over the world (Tester and Davenport, 2003). In the light of predicted population growth to 9.3 Billon by 2050 (Lee, 2011), global food requirements are expected to increase by 70 to 110% (Tilman et al., 2011). Wheat is one of the most important crops which providing nearly 55% of the consumed carbohydrates world-wide (Gupta et al., 1999) but is not highly salt tolerant, and its commercial production is substantially reduced as the soil salinity level rises to 100 mM NaCl and is not possible in soils containing more than 250 mM NaCl (Munns et al., 2006; Munns and Tester, 2008). Thus, improving salinity stress tolerance in wheat is an urgent task to cope with the possible shortage of food supply in the near future. This is especially true for the hexaploid bread wheat that composes about 95% of all wheat grown world-wide (Shewry, 2009).

Sodium uptake and sequestration has always been under spotlight of researchers aimed at finding the traits or genes which can be selected to improving salinity tolerance in wheat. Early studies using $^{22}Na^+$ isotopes showed that salt tolerant wheat variety have significantly lower Na^+ accumulation in the shoot (Davenport et al., 1997), suggesting an efficient Na^+ exclusion mechanism. The following studies by Munns and colleagues (Munns et al., 2002, 2003, 2006; Munns and James, 2003; Lindsay et al., 2004) suggested that targeting Na^+ exclusion from shoot was a promising way to improving salinity tolerance in this species. Indeed, under saline condition, flag leaf Na^+ was significantly reduced from 326 mM in commercial variety Tomaroi to 87 mM in transformed Tamaroi plants that expressed *TmHKT1;5-A* gene enabling Na^+-retrieval from the xylem (Munns et al., 2012). This has resulted in about 20% increase in wheat yield under saline field conditions (from 1.30 to 1.61 tonnes per hectare). Using microelectrode ion flux measuring MIFE technique, Cuin et al. (2011) found that Kharchia 65 (accepted as a "standard" for salinity tolerance in wheat by most breeders) had the highest root Na^+ extrusion ability compared with other seven wheat varieties studied. Pharmacological experiments and experiments with transgenic Arabidopsis mutants have confirmed that this Na^+ efflux was mediated by the plasma membrane SOS1 Na^+/H^+ antiporter. Similar studies conducted on sorghum (Yang et al., 1990), maize (Fortmeier and Schubert, 1995),

and tomato (Al-Karaki, 2000) have also suggested that plant's ability to exclude Na^+ from uptake in these species was positively correlated with the overall salinity tolerance. Even in lower plants the ability to avoid the accumulation of Na^+ in cytosol is critical for its salinity tolerance (e.g. cyanobacteria; Allakhverdiev et al., 2000; Allakhverdiev and Murata, 2008).

The above beneficial effects of Na^+ exclusion from uptake was always attributed to its toxic effect on cell metabolism (Maathuis and Amtmann, 1999; Munns and Tester, 2008) and essentiality to maintain low level of Na^+ in the cytosol. However, the same goal may be achieved by the efficient Na^+ sequestration in the vacuole. The latter trait is commonly employed by halophytes (naturally salt-loving plants; Flowers and Colmer, 2008; Shabala and Mackay, 2011; Shabala, 2013; Bonales-Alatorre et al., 2013a, b), and some evidences were presented that salt-tolerant wheat varieties may also possess better vacuolar Na^+ sequestration ability (e.g. Saqib et al., 2005b). However, most of these studies were conducted on leaves, while the role of Na^+ sequestration in roots received less attention. In *Thellungiella salsuginea*, a halophytic relative of *Arabidopsis thaliana*, Oh et al. (2009) showed that vacuoalr Na^+ fluorescent intensities in cortex cells of root tip region is higher in *thsos1-4* than wild type. However, to the best of our knowledge, the issue of tissue-specificity of vacuolar Na^+ sequestration between different root zones, and its link with the overall salinity tolerance, has never received a proper attention, neither in wheat nor in any other crop species.

The root anatomy and functional structure can be generally divided into four different zones: (1) root meristem, (2) the distal elongation (or transition) zone, (3) elongation zone, and (4) mature zone (Verbelen et al., 2006; Baluška and Mancuso, 2013). So far, most studies of Na^+ distribution in plant roots under salt stress was conducted either at the level of whole root (e.g. Matsushita and Matoh, 1991; Flowers and Hajibagheri, 2001; Rus et al., 2001), or were focused on cell-type-specific Na^+ distribution in roots (Huang and van Steveninck, 1988; Storey et al., 2003; Oh et al., 2009, 2010). While these and some other (Li et al., 2012; Cuin et al., 2011) papers showed a heterogeneity of Na^+ distribution within the root, none of them discussed the difference in Na^+ patterning between intracellular compartments within functionally different root zones. In *Thellungiella salsuginea*, Na^+ accumulated inside the pericycle in *thsos1-4* mutant, while in the wild type it was confined in vacuoles of epidermal and cortical cells (Oh et al., 2009). Cell-type-specific Na^+ distribution patterns in hypodermis, cortex, endodermis, and pericycle were also studied in salinized grapevines using X-ray microscopy method

(Storey et al., 2003). Here, vacuolar Na^+ was sequestered predominantly in endodermis and pericycle cells. However, to the best of our knowledge, no study has compared Na^+ distribution between cytosol and vacuole in functionally different root zones within the same tissue, at least in bread wheat.

In the present work, variability of vacuolar Na^+ sequestration in four different root zones under salt stress was studied using six bread wheat varieties contrasting in their salinity tolerance. Cytosolic and vacuolar Na^+ content in different root zones was quantified by CoroNa Green fluorescent dye imaging, and the link between tissue-specific vacuolar Na^+ sequestrations in specific root zone and the overall salinity stress tolerance was explored. We report that the overall salinity stress tolerance correlates positively with vacuolar Na^+ sequestration ability in the mature root zone but not in the root apex. At the same time, cytosolic Na^+ levels in root meristem were significantly higher in salt tolerant than sensitive group, suggesting that meristem cells may play a role of the "salt sensor". The overall Na^+ accumulation was highest in the elongation zone, suggesting its role in osmotic adjustment and turgor maintenance required to drive root expansion growth.

7.2 Materials and methods

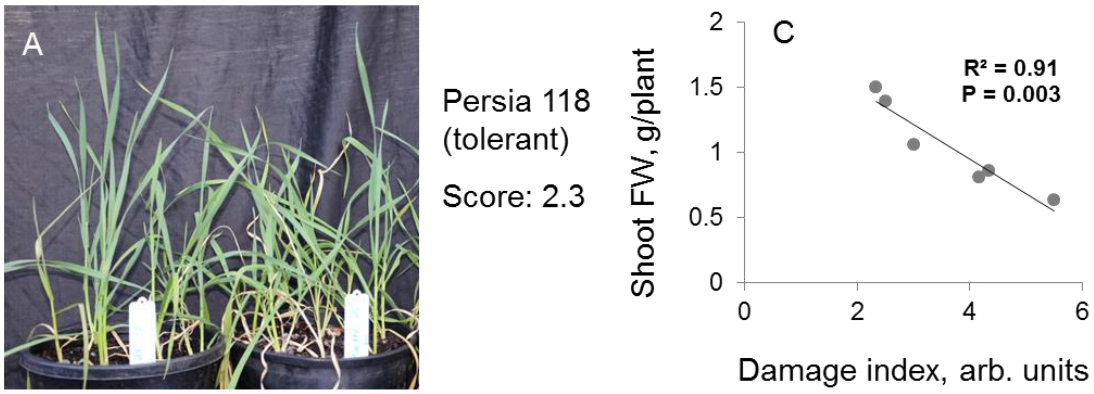
7.2.1 Plant materials and growth conditions

Six bread wheat (*Triticum aestivum*) varieties contrasting in their salinity tolerance (tolerant - Persia 118, Cranbrook, and Westonia; sensitive - Iran 118, Belgrade 3, and 340) were used in this study. All of the tolerant and sensitive bread varieties used in this study were chosen according to their grain yield (Cuin et al., 2009; Zhu et al., 2016). All seeds were obtained from the Australian Winter Cereals Collection and multiplied in our laboratory. Plants were grown in February-March 2013 in the glasshouse facilities at the University of Tasmania essentially as described in Chen et al. (2007d). 12 seeds for each variety were sown in 4.5 L PVC pots with the standard potting mix by triplicates. After emerging (roughly 6 days), salt treatment (300 mM NaCl) were applied for about five weeks. Plants were irrigated twice per day by an automatic watering system with dripper outlets, and were uniformly thinned to eight plants in each pot after roughly 10 days sowing. A saucer was placed under each pot. For confocal imaging experiments, seeds were sterilized with 5% commercial bleach for 15 min, and then washed thoroughly by the running tap water for a half hour. Seeds were then germinated in wet paper rolls in growth chambers at 23 ± 1°C (16 h light/ 8 h dark regime). Four days old wheat roots

were treated with 100 mM NaCl for 72 h, and then stained by CoroNa Green dye for the LSCM (laser scanning confocal microscopy) measurements as described below.

7.2.2 Whole-plant performance assessing

Before harvesting, plants "damage index" were scored on zero to 10 scale (0 – no visual symptoms of stress; 10 – dead plants; Supplementary Fig. S1). The higher damage index score represents the lower salt tolerance. Then, the stem was cut 1 cm above the ground, and shoot fresh weight (FW) was measured.



Suppl. Fig. S1. Quantifying salinity tolerance in bread wheat by the damage index. The extent of salinity damage to plants was quantified on 0 (no visual symptoms of stress) to 10 (all plants are dead) scoring scale. Two examples – for tolerant variety Persia 118 (A) and sensitive variety 340 (B) – are shown. C – a correlation between damage index and shoot fresh weight in six bread wheat treated with 300 mM NaCl for about 5 weeks. Each point represents a variety.

7.2.3 Confocal laser scanning microscopy measurements

Measurements of cytosolic and vacuolar Na^+ content in wheat root cells using the green fluorescent Na^+ dye CoroNa Green acetoxymethyl ester were essential as described in Bonales-Alatorre et al. (2013b). The dye has absorption and fluorescence emission maxima of approximately 492 and 516 nm, respectively. The dye was reconstituted as a stock with anhydrous dimethyl sulfoxide before use. The CoroNa Green indicator stock was added to 5 mL of measuring buffer (10 mM KCl, 5 mM Ca^{2+}-MES, pH 6.1) and diluted to a final concentration of 15 mM. Ten millimetres-long root segments were cut from the apical (the first 10 mm from the apex) and mature (30 to 40 mm from the apex) root zones and incubated for 2h in the dark in a solution containing 20μM CoroNa Green.

After incubation, the samples were rinse in a buffered MES solution (pH 6.1) and examined using confocal microscopy. Confocal imaging was performed using an upright Leica Laser Scanning Confocal Microscope SP5 (Leica Microsystems, Germany) equipped with a 40× oil immersion objective. To analyse sodium intracellular localisation CoronaNa Green AM (Molecular Probes, USA) was used. The excitation wavelength was set at 488 nm, and the emission was detected at 510-520 nm. Comparison of different levels of fluorescence between cells was carried out by visualizing cells with the identical imaging settings of the confocal microscope (i.e. exposure times, laser intensity, pinhole diameter and settings of the imaging detectors). Images were analysed with LAS Lite software. Six to 8 individual roots (each from different plant) were used; and at least 2 images were taken for each root zone. For analysis, several lines were drawn across the so-called "region of interest" (ROI; Fig 1A) in an appropriate root zone. Continuous fluorescence intensity distribution profiles (quantified in arbitrary units by LAS software) were then obtained and plotted in an Excel file (Fig 1B). The mean fluorescence intensity values for cytosolic and vacuolar compartments were then calculated for each cell by attributing signal profiles to root morphology (visualised by light microscopy images). The data was then averaged for all cells measured for the same treatment. The background signal was measured from the empty region and then subtracted from the readings, to obtain corrected fluorescence values. Depending on the root zone, readings from between 70 and 300 cells were averaged and reported for each genotype.

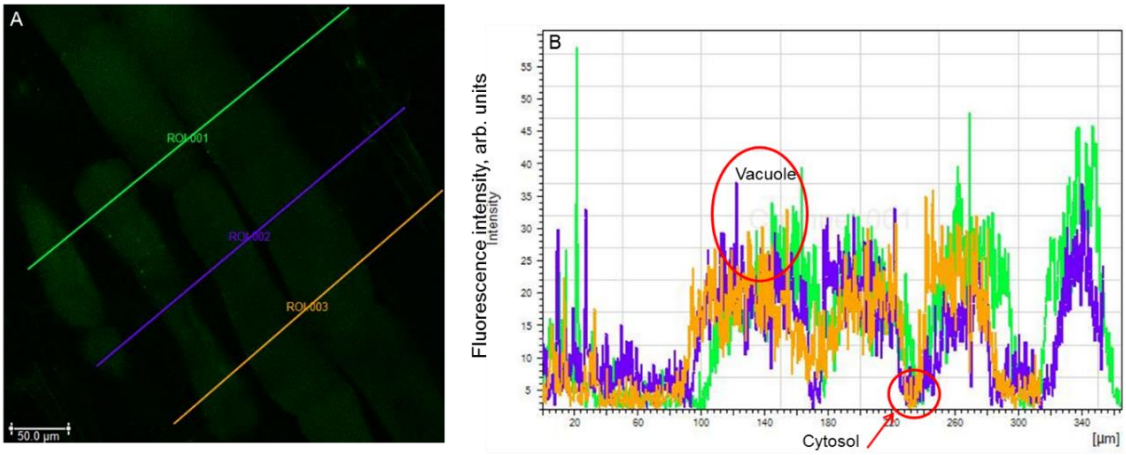


Fig. 1. Illustration of the quantification procedure for Na^+ distribution between the cytosol and the vacuole. (A) - Several lines are drawn across the so-called "region of interest" (ROI) in an appropriate root zone. (B) - Continuous fluorescence intensity distribution profiles obtained by LAS software. The mean fluorescence intensity values for cytosolic and vacuolar compartments are then calculated for each cell by attributing signal profiles to root morphology (visualised by light microscopy images; not shown in a figure). The shown image was obtained from mature region of Persia 118 cultivar. The first 100 mm in (B) shows the Na^+ fluorescence of the vessel cell.

A further validation of the above protocol was conducted using roots co-stained with CoroNa Green-AM and FM4-64, a dye that stains both plasma and vacuolar membranes (Oh et al 2010; Bassil et al. 2011) and allow a better resolution between intracellular compartments. After 1h incubation of 20µM CoroNa Green-AM (as described above), the same root samples were then incubated together with 20µM FM4-64 for another 1 h to visualize tonoplast. Then, roots were rinsed with buffer solution (10 mM KCl, 5 mM Ca^{2+}-MES, pH 6.1) for 3 min for confocal imaging. For FM4-64 fluorescence, the 488-nm excitation line was used and collected with a 615-nm long-pass filter. Results of this experiment are illustrated in Fig 2 showing intracellular Na^+ distribution in the transition zone of cultivar Persia 118. Panel A shows distribution of CoroNa Green and FM4-64 in co-stained root samples, while panel B and C shows the same root stained with FM4-64 and CoroNa Green, respectively. Two cells having multiple vacuoles (circled) were then selected for analysis (depicted in panels D and E). Two ROI lines were then drawn crossing two vacuoles in each of the cells. Intracellular Na^+ distribution was then quantified (panel F). As one can see, two major peaks in each cell correspond to two vacuoles, while three troughs report values for cytosolic Na^+.

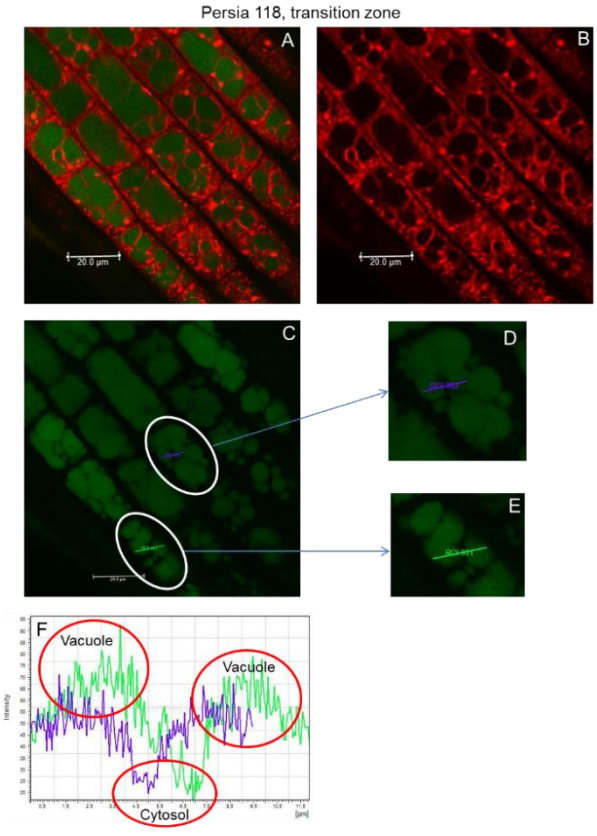


Fig. 2. Quantifying intracellular Na^+ distribution between the cytosol and the vacuole by double staining procedure. Intracellular Na^+ distribution is illustrated using the transition zone of cultivar Persia 118 as an example. (A) The root transition zone co-stained with Corona Green AM and FM4-64. The same root stained with FM4-64 dye (B) and CoroNaGreen (C). Two cells having multiple vacuoles (circled) were then selected for analysis (depicted in D, E). Two lines were then drawn across the "region of interest" (ROI) for each of the chosen cells in (C) (also shown in D and E, separately), crossing two vacuoles in each of cells. Intracellular Na^+ distribution of the selected cells in C, D, and E was then quantified (F).

7.2.4 Statistical analysis

All data (given as mean ± SE, n = sample size) were analysed by using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). All of the replicates are biological replicates. Comparison of cytosolic or vacuolar Na^+ fluorescent intensity between different varieties in root zones was done by one-way ANOVA based on Duncan's multiple range test. Different lowercase letters represent significant difference between varieties. Data with same lowercase letters are not significantly different at $P < 0.05$. Comparison of cytosolic or vacuolar Na^+ fluorescent intensity between tolerant and sensitive group in different root zones was done by independent samples *t*-test. The significance levels are $*P < 0.05$, $**P < 0.01$and $***P < 0.001$. The significance of correlations between different parameters was determined by Bivariate Correlations based on two-tailed Pearson Correlation. The data used for correlation analysis are the average values of measured independent parameters for each variety.

7.3 Results

7.3.1 Whole-plant performance

Bread wheat varieties used in this study (three tolerant and three sensitive varieties were used (Cuin et al., 2009; Zhu et al., 2016)) showed big variability in salinity stress tolerance. Salinity damage index (a measure of salt tolerance; see Fig. S1A, S1B for details) ranged from the highest (most sensitive) 5.5 ± 0.5 in variety 340 to the lowest (most tolerant) 2.3 ± 0.4 in variety Persia 118 (significant at $P < 0.01$; Fig. 3A). Similarly, the highest shoot fresh weight (FW) was found in Persia 118 (1.5 ± 0.1 g/plant), while variety 340 showed the lowest shoot FW (0.6 ± 0.2 g/plant) (Fig. 3B). Accordingly, all varieties were grouped into tolerant and the sensitive clusters (Fig. 3C, D). The tolerant cluster showed much less damage (about 2-fold; $P < 0.05$, Fig. 3C) and 70% higher shoot biomass (significant at $P < 0.05$, Fig. 3D) compared with the sensitive cluster. A significant negative correlation ($r = 0.95$, $P < 0.01$) was found between shoot FW and damage index among all varieties (Fig. S1C).

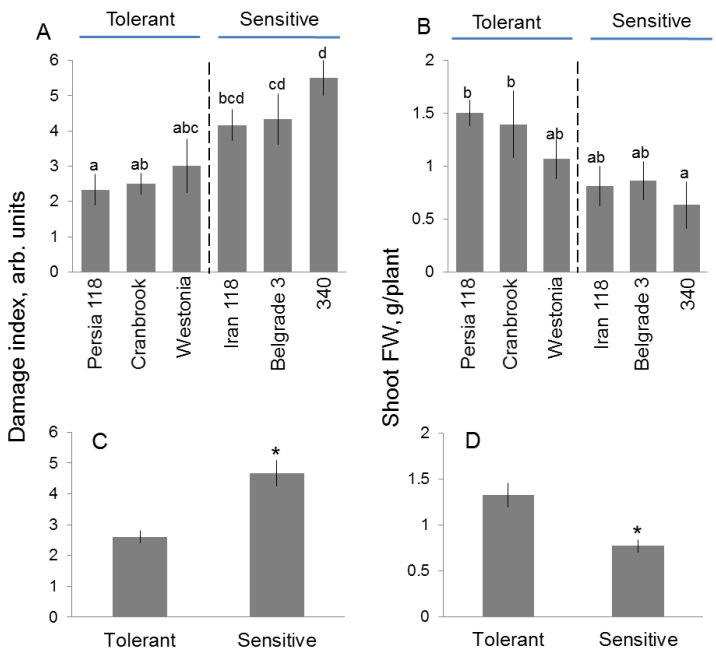


Fig. 3. Genetic variability of salinity stress tolerance in bread wheat.
Damage index (A) and shoot fresh weight (B) of six bread wheat cultivars contrasting in their salinity tolerance after 38 days of 300 mM NaCl treatment in glasshouse. Different lowercase letters represent significant difference between varieties at P < 0.05. C, D – average pooled data for tolerant and sensitive clusters shown in A & B. Mean ± SE (n= 3; 24 plants in total, eight plants in each pots). Asterisk indicates significant difference between clusters at $P < 0.05$. Note: The salt tolerance of used varieties is based on their grain yield (Cuin et al., 2009; Zhu et al., 2016).

7.3.2 Sodium accumulation profiles

Before cell- and genotype-specific Na^+ distribution was quantified, a series of methodological experiments was conducted to eliminate possible confounding effects of dye loading and stress-induced changes in intracellular ionic conditions on fluorescence measurements. First, CoroNa Green was calibrated in a cytosol–like solution (50 to 100 mM K^+; < 1 mM Ca^{2+}; pH = 7.2) in a broad range of Na^+ concentration (1, 3, 10, 30 and 100 mM) in *in vitro* experiments. As shown in Fig 4, a 2-fold drop in background K^+ concentration from 100 to 50 mM (mimicking NaCl-induced reduction in cytosolic K^+ under stress conditions; Shabala et al. 2006) had no significant ($P < 0.05$) impact on fluorescence signal except the lowest (1 mM NaCl) concentration, which is well-below expected levels for cytosolic Na^+ (Munns and Tester, 2008). Calibration characteristics were also insensitive to pH in physiological (5 to 7.2) pH range (data not shown). Thus, the possible difference in K^+ retention ability or stress-induced changes in intracellular pH between genotypes had no confounding effects on CoroNa Green readings. Dye loading profiles were uniform between various Z-plains (Suppl Fig S2) suggesting that 2 h of loading was sufficient to ensure its homogenous uptake by most cells.

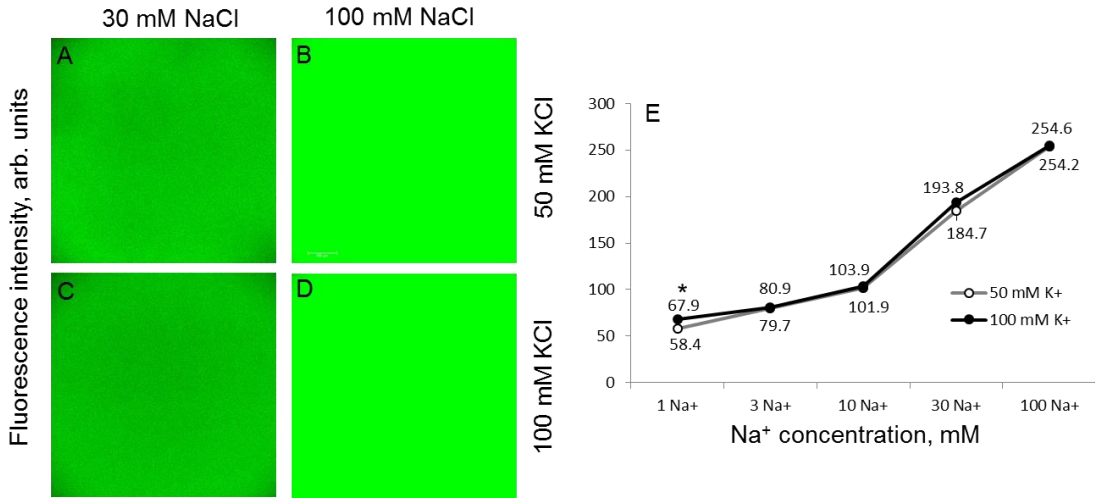
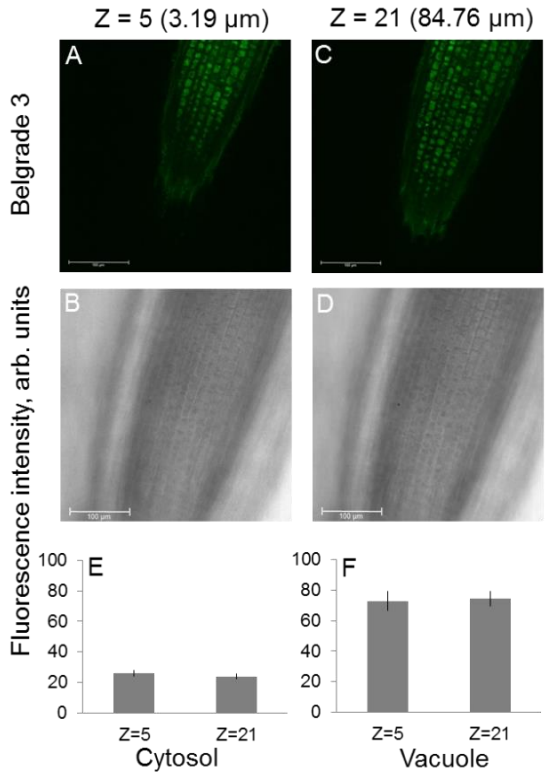


Fig. 4. Methodological aspects of CoroNa Green dye calibration. CoroNa Green dye was calibrated *in vitro* in a buffer solution containing different Na^+ and K^+ concentration. (A -D) – *in vitro* fluorescence images for two Na^+ and two K^+ concentrations used in experiment. One (of four) representative images is shown for each Na^+ and K^+ concentration; (E) – dose-dependency of Na^+ fluorescence signals at 50 mM and 100 mM KCl background. Mean ± SE (n = 60; 4 images, 15 replicates recorded from each image). Asterisk indicates a significant (at $P < 0.05$) difference between K^+ treatments. Please note that in most cases the error bar is smaller than the symbol *per se*. Note: The relationship between the applied Na^+ and detected Na^+ intensity is still non-linear even the X axis was changed to a linear scale.



Suppl. Fig. S2. Homogeneity of CoroNa Green fluorescence signal between various cell layers.

(A, C) – Representative (one of 4) images of CoroNa Green fluorescence in the root apex of bread wheat cultivar Belgrade 3 taken at different focal depth (A – top cell layer; C – fifth cell layer; ~80 □m deeper inside the root). (B, D) – Respective light images. (E, F) - Mean fluorescence intensity values in cytosol (E) and vacuole (F) of cells in the first (shown in A) and fifth (shown in C) cell layers. Mean ± SE (n = 20-24). The difference between cell layers (different Z-plains) is not significant at $P < 0.05$, neither in vacuole nor in the cytosol.

Plants grown in Na-free solution (Milli-Q water) showed negligible small Na^+ fluorescence signals (Fig 5) while root exposure to 100 mM NaCl for 72 h resulted in massive accumulation of Na^+ in root tissues (Fig. 5).

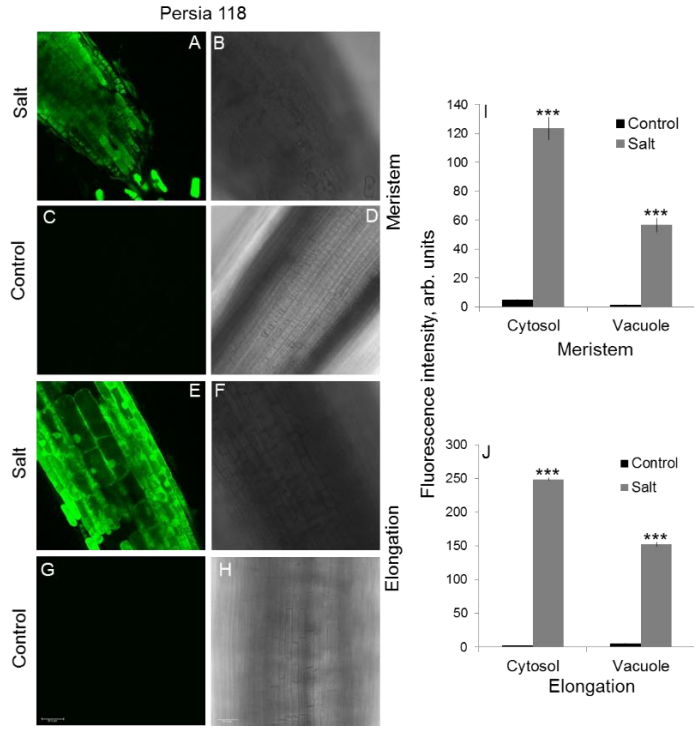


Fig. 5. The comparison of Na^+ fluorescence intensity in salt-grown and Na-free roots.
(A, C, E, G) - Na^+ fluorescence intensity was measured in root of Persia 118 cultivar grown in the presence (100 mM NaCl for 72 h) and absence (milliQ water) of Na^+ in the growth media. One (of 12) representative images is shown for each zone. (B, D, F, H) - Light images on the appropriate slides shown on the left. (I, J) – Quantification of cyotosolic and vacuolar Na^+ in salt-grown and Na-free roots. Mean ± SE (n = 42-96 cells from 6 roots).

When the method was applied to all salt-grown plants, sodium distribution within the root showed a clear pronounced tissue- and genotype-specificity. The representative images for one tolerant (Persia 118) and one sensitive (Iran 118) variety for each of four measured zones (meristem, transition, elongation, and mature zone) are shown in Fig. 6, and the average fluorescence intensity of Na^+ in cell vacuole and cytosol in each variety are quantified in Figs. 7-10.

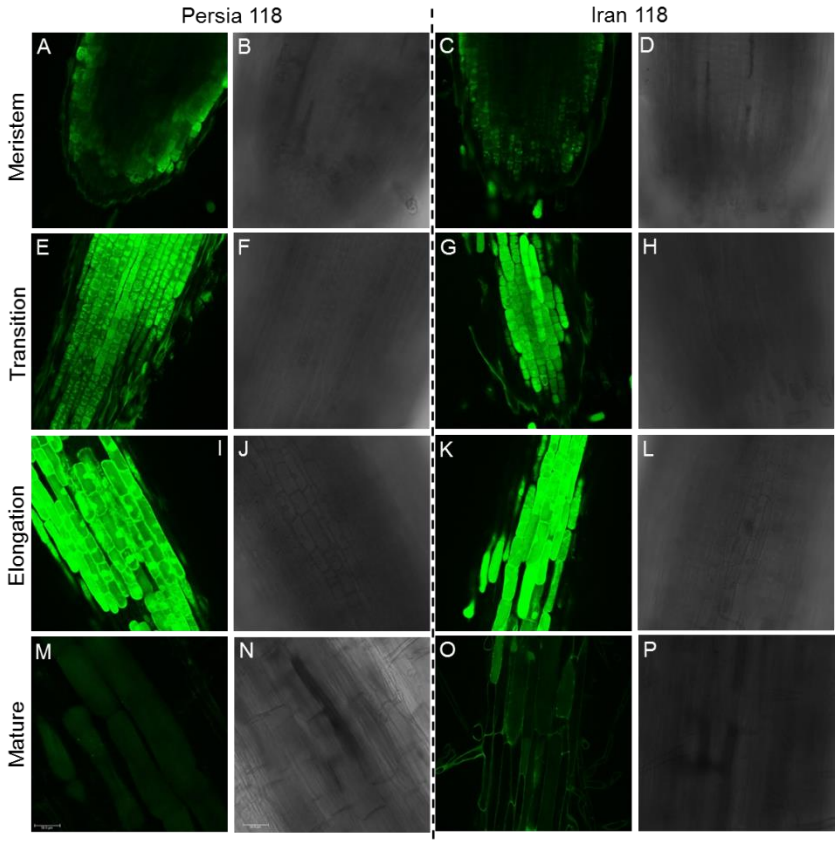


Fig. 6. Na^+ accumulation profiles in bread wheat visualised by CoroNa Green dye imaging.
The representative images of Na^+ distribution within one tolerant (Persia 118) and one sensitive (Iran 118) varieties are shown for four different root zones: meristem (A, C); transition zone (E, G); elongation zone (I, K), and mature zone (M, O). One of 12 representative images is shown for each zone. Panels next to fluorescence images represent respective light images (B, D, F, H, J, L, M, P).

7.3.3 Root meristem

Significantly higher quantities of Na^+ were accumulated in the cytosol of meristematic cells in a tolerant compared with sensitive cluster (Fig. 7). Here, cytosolic Na^+ intensity ranged from highest 179.9 ± 7.7 (in tolerant Westonia) to lowest 20.3 ± 1.2 (in sensitive Belgrade 3), declining in a sequence Westonia > Cranbrook > Persia 118 > Iran 118 > 340 > Belgrade 3 (Fig. 7A). Overall, the amount of Na^+ stored in cytosol of cells in the meristem region of the tolerant cluster was 4.3-fold higher compared with the sensitive cluster (159.2 ± 17.9 vs 37.0 ± 8.4; $P < 0.01$; Fig. 7C) and also significantly ($P < 0.05$) higher than vacuolar Na^+ content (Fig. 7A,B; Suppl. Fig. S3). At the same time, vacuolar Na^+ intensity in root meristem zone ranged from the highest 108.9 ± 4.4 (in sensitive Iran 118) to lowest 56.7 ± 4.9 (in tolerant Persia 118), declining in a sequence Iran 118 > 340 > Cranbrook > Westonia > Belgrade 3 > Persia 118 (Fig. 7B; Suppl. Fig. S3). Overall, no significant (at $P < 0.05$ level) difference was found in vacuolar Na^+ in meristem cell vacuoles among two contrasting clusters (Fig. 7D). Furthermore, a significant ($P = 0.05$) negative correlative relationship ($r = 0.81$) was found between cytosolic Na^+ intensity in root meristem zone and salinity-induced damage index (Suppl. Fig. S4A), while a weak positive correlation ($r = 0.47$, $P > 0.05$) was found between the vacuolar Na^+ intensity in root meristem zone and the damage index (Suppl. Fig. S4E).

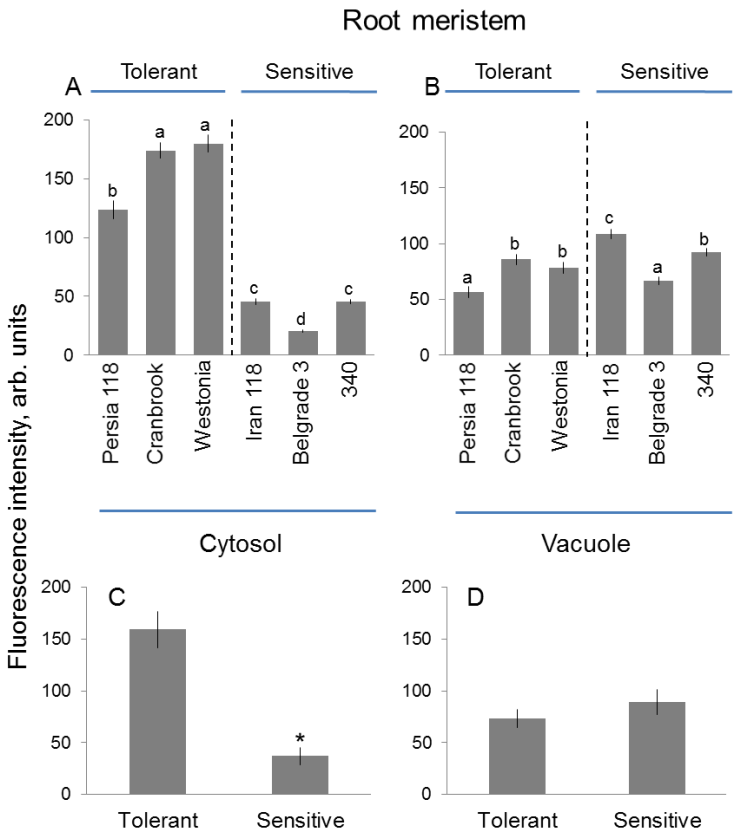
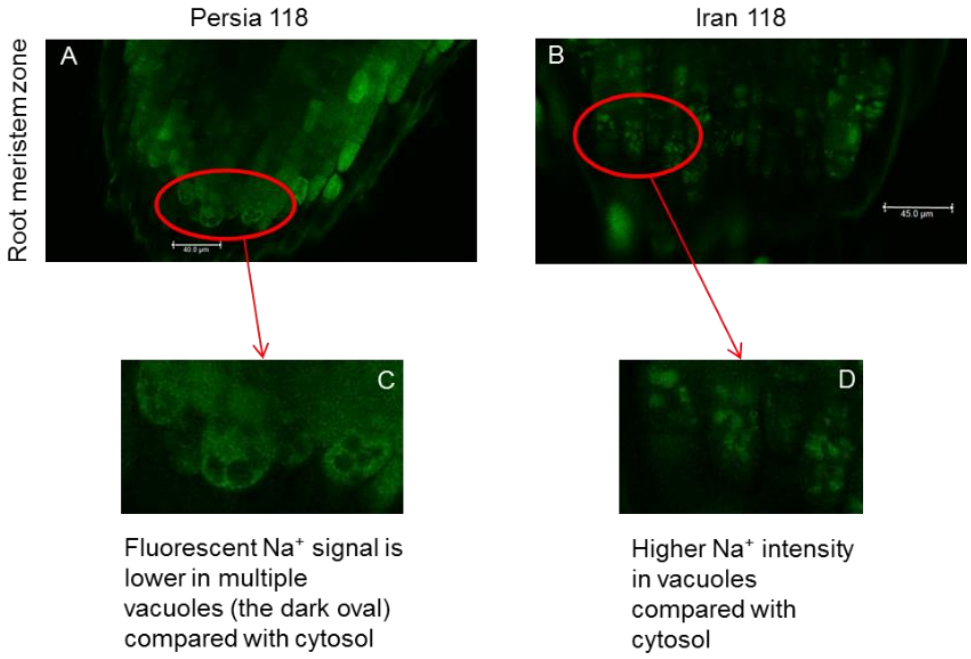
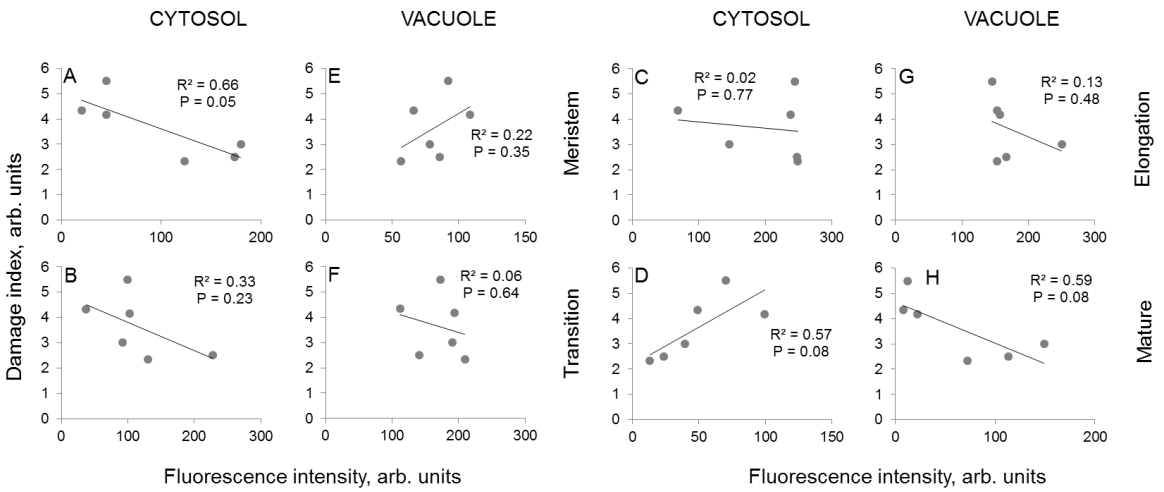


Fig. 7. Na^+ accumulation and patterning between cytosol and vacuole in meristematic root zone in bread wheat genotypes.

Intensity of CoroNa Green fluorescence in cytosolic (A) and vacuolar (B) compartments (arb. units) in root meristem of six bread wheat varieties contrasting in their salinity tolerance. Mean ± SE (n= 72-96 cells from at least 6 individual plants). Different lowercase letters represent significant difference between varieties at $P < 0.05$. C, D – averaged pooled values for cytosolic (C) and vacuolar (D) Na^+ intensity for salt-tolerant and salt-sensitive clusters shown in A & B. Mean ± SE (n = 215 to 300; 3 varieties x 72-96 cells analysed for each variety). Asterisk indicates significant difference between clusters at $P < 0.01$.



Suppl. Fig. S3. Na^+ distribution between the cytosol and the vacuole in meristematic zone of wheat root. A representative images of the root meristem loaded with CoronaGreen AM is shown for salt-tolerant cultivar Persia118 and salt-sensitive cultivar Iran118. As one can see, in tolerant variety most of the Na^+ is located in the cytosol while vacuoles are dark and show not much fluorescent signal. The oppositeis true for salt-sensitive genotype.



Suppl. Fig. S4. Correlation between salinity stress tolerance and Na^+ distribution in cytosol and vacuole in different root zones. Correlation between salinity stress tolerance (quantified as a damage index) and cytosolic (A-D) and vacuolar (E-H) Na^+ intensities in four functional root zones. Each point represents a variety.

7.3.4 Transition zone

Vacuolar Na^+ intensity in the root transition zone was higher than cytosolic Na^+ intensity, in both tolerant and sensitive clusters (illustrated in Fig. 5E, G and quantified in Fig. 8). Belgrade 3 showed the lowest (37.6 ± 2.3) cytosolic Na^+ intensity in root transition zones, while Cranbrook showed the highest (227.7 ± 5.0) (Fig. 8A). Highest vacuolar Na^+ intensity was reported for Persia 118 (209.5 ± 6.2), and the lowest for Belgrade 3 (111.9 ± 5.8) (Fig. 8B). Overall, no clear trends were observed in Na^+ distribution between cytosol and vacuole in the root transition zone. As a result, no significant difference in either cytosolic or vacuolar Na^+ intensity was found between salt tolerant and sensitive clusters here (Fig. 8C, D). Only very modest ($r = 0.57$, $P = 0.23$) negative correlation was found between cytosolic Na^+ intensity and salt damage index (Suppl. Fig. S4B), while no correlation was found between cytosolic Na^+ intensity in root elongation zone and damage index ($r = 0.14$, Suppl. Fig. S4C). Please note: For quantifying the Na^+ intensity in cytosol and vacuole in root meristem and transition zone, FM4-64 dye which can specifically stain the tonoplast was used to visualize the boundary between the small vacuoles (as shown in Fig. 2).

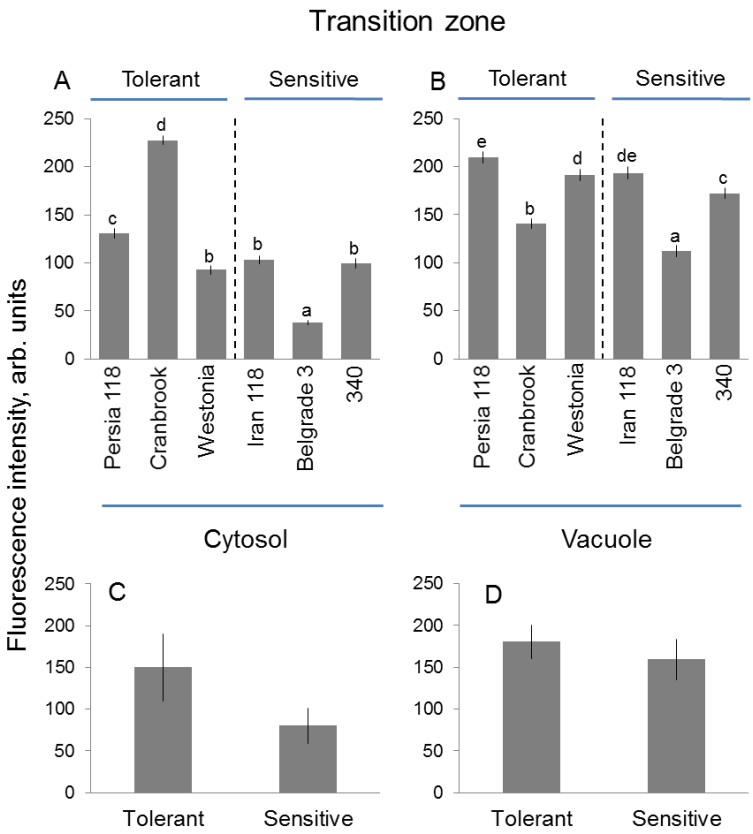


Fig. 8. Na^+ accumulation and patterning between cytosol and vacuole in transition root zone in bread wheat genotypes. Intensity of CoroNa Green fluorescence in cytosolic (A) and vacuolar (B) compartments (arb. units) in transition root zone of six bread wheat varieties contrasting in their salinity tolerance. Mean ± SE (n= 72-96 cells from at least 6 individual plants). Different lowercase letters represent significant difference between varieties at P<0.05. C, D – averaged pooled values for cytosolic (C) and vacuolar (D) Na^+ intensity for salt-tolerant and salt-sensitive clusters shown in A & B. Mean ± SE (n = 215 to 300; 3 varieties x 72-96 cells analysed for each variety).

7.3.5 Elongation zone

Cells in root elongation zone had slightly higher amounts of Na^+ in the cytosol compared with vacuoles (Fig. 5I, K), both in tolerant and sensitive clusters (Fig. 9). Belgrade 3 showed the lowest cytosolic Na^+ intensity (68.4 ± 4.7), while highest values were reported for Persia 118 (248.9 ± 2.0) (Fig. 9A). Vacuolar Na^+ intensity was highest in tolerant Westonia (250.4 ± 1.7) and lowest in sensitive variety 340 (145.4 ± 3.3) (Fig. 9B). Overall, no clear trends were observed in Na^+ distribution between cytosol and vacuole in root elongation zone in six varieties (Fig. 9), and no significant difference in either cytosolic or vacuolar Na^+ intensity was found between two contrasting clusters (Fig. 9C, D). No significant (at $P < 0.05$ level) correlation was found between the damage index and cytosolic Na^+ intensity in root elongation zone (r = 0.14, Suppl. Fig. S4C), as well as for vacuolar Na^+ intensity (r = 0.36, $P = 0.48$) (Suppl. Fig. S4G).

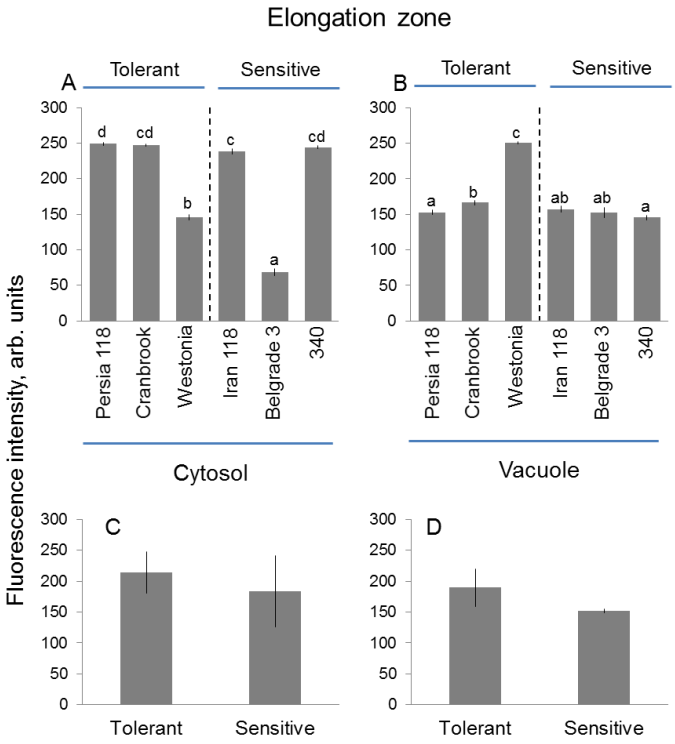


Fig. 9. Na^+ accumulation and patterning between cytosol and vacuole in elongation root zone in bread wheat genotypes. Intensity of CoroNa Green fluorescence in cytosolic (A) and vacuolar (B) compartments (arb. units) in root elongation zone of six bread wheat varieties contrasting in their salinity tolerance. Mean ± SE (n= 72-96 cells from at least 6 individual plants). Different lowercase letters represent significant difference between varieties at P<0.05. C, D – averaged pooled values for cytosolic (C) and vacuolar (D) Na^+ intensity for salt-tolerant and salt-sensitive clusters shown in A & B. Mean ± SE (n = 215 to 300; 3 varieties x 72-96 cells analysed for each variety).

7.3.6 Mature zone

Cytosolic Na^+ in root mature zone was significantly lower in salt-tolerant compared with salt- sensitive cluster while for vacuolar Na^+ this trend was inverse (Fig. 6M, O ; Fig 10). Another important trend was that the overall amount of Na^+ accumulated in the cytosol was much less compared with any other zones (Fig. 6). Cytosolic Na^+ intensity in root mature zone was highest in sensitive Iran 118 (99.6 ± 15.1) and lowest in tolerant Persia 118 (13.1 ± 3.5) (Fig. 10A). Salt-tolerant cultivar Westonia had the highest vacuolar Na^+ intensity (149.0 ± 19.0), while salt-sensitive Belgrade 3 had the lowest (7.6 ± 1.3) (Fig. 10B). On average, cytosolic Na^+ intensity in mature root zone was 3-fold lower in tolerant (25.4 ± 7.7) than sensitive (73.0 ± 14.6) (Fig. 10C; significant at $P < 0.05$). At the same time, vacuolar Na^+ intensity in mature root zone in tolerant group was 8-fold higher compared with sensitive group (111.4 ± 22.2 vs 13.9 ± 4.3; significant at *P*

< 0.01; Fig. 10D). Overall, a positive relationship (r = 0.76, *P* = 0.08) was found between plant damage index and cytosolic Na^+ intensity in mature root zone (Suppl. Fig. S4D), while for vacuolar Na^+ intensity this correlation was negative (r = 0.77, *P* = 0.08; Suppl. Fig. S4H).

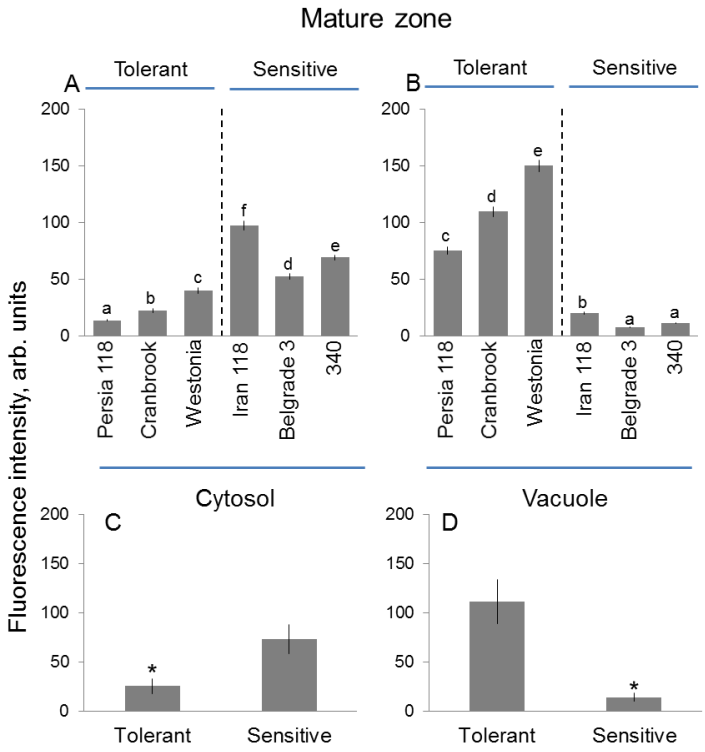


Fig. 10. Na^+ accumulation and patterning between cytosol and vacuole in mature root zone in bread wheat genotypes.
Intensity of CoroNa Green fluorescence in cytosolic (A) and vacuolar (B) compartments (arb. units) in mature root zone of six bread wheat varieties contrasting in their salinity tolerance. Mean ± SE (n= 72-96 cells from at least 6 individual plants). Different lowercase letters represent significant difference between varieties at P<0.05. C, D – averaged pooled values for cytosolic (C) and vacuolar (D) Na^+ intensity for salt-tolerant and salt-sensitive clusters shown in A & B. Mean ± SE (n = 240 to 320; 3 varieties x 72-96 cells analysed for each variety). Asterisk indicates significant difference between clusters at P < 0.05.

7.4 Discussion

7.4.1 Vacuolar Na^+ sequestration in mature root zone but not root apex correlates with salinity tolerance in bread wheat

For glycophytes such as bread wheat, Na^+ is not considered to be an essential nutrient (Maathuis, 2014) and, when present in excessive quantities in soil, leads to cytosolic Na^+ toxicity, and impairs plant growth. Maintenance of the optimal cytosolic Na^+ level under saline conditions requires effective extrusion of Na^+ from the cytosol, either back to external media, or into vacuole (Maathuis and Amtmann, 1999; Blumwald, 2000).

In the present work, we have investigated Na^+ distribution between the cytosol and the vacuole in four different root zones in contrasting bread wheat varieties exposed to salinity stress. Surprisingly, vacuolar Na^+ sequestration was correlated positively with salinity tolerance *only* in mature root zone. In a contrast, no significant difference in vacuolar Na^+ sequestration pattern was found between salt tolerant and sensitive clusters in either of other three zones: transition (Fig. 8B, D), elongation (Fig. 9C, D), or meristem

(Fig. 7 C, D). At the same time, significantly lower cytosolic Na^+ intensity was found in salt tolerant compared with sensitive cluster in mature zone (Fig. 10A, B). Taken together these results suggest that the ability of mature root cell vacuoles to sequester excessive Na^+ is one of the key determinants of salinity tolerance in bread wheat.

It should be noted that, given the non-linearity of the calibration curve (Fig. 4), the fluorescent intensities measured might not linearly relate to the absolute sodium concentrations in plant tissues, so some caution is needed while interpreting the presented data in quantitative terms.

We have used term "surprisingly" above because root apex is a house for most metabolically active cells and, as such, has often considered as a potential target for many abiotic stresses such as Al toxicity (Doncheva et al., 2005), oxidative stress (Demidchik et al., 2007), and heavy metal toxicity (Halušková et al., 2009). It was also unexpected as SOS1 Na^+/H^+ exchangers removing Na^+ from uptake are believed to be expressed predominantly in the root apex (Shi et al., 2002). Given the fact that the functional expression of SOS1 exchangers was always considered as an important component of salinity tolerance trait (Zhu, 2003; Apse and Blumwald, 2007; Olías et al., 2009b; Oh et al., 2009), the fact that cytosolic Na^+ levels in root meristem of tolerant group was 6-fold higher compared with mature zone (159.2 ± 17.9 vs 25.4 ± 7.7, respectively; Fig. 11) was unexpected.

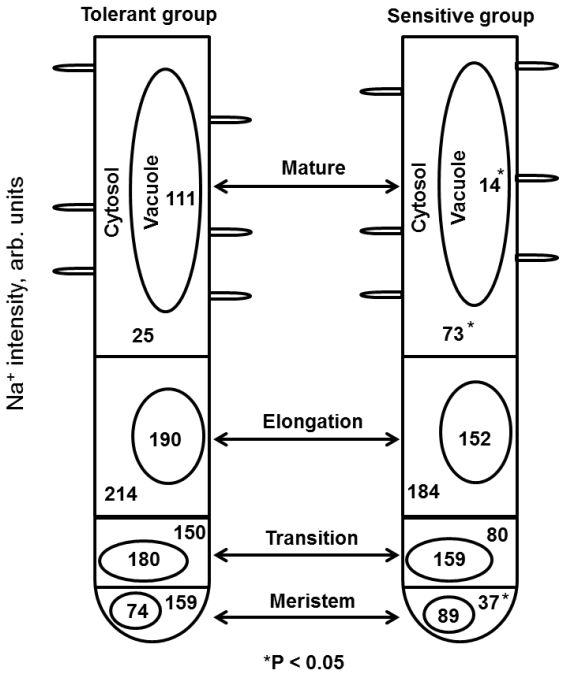


Fig. 11. A summary of Na^+ distribution between various intracellular compartments and functionally different root zones in bread wheat. Numbers represent mean pooled values for CoroNa Green fluorescence intensity (arbitrary units) for salt-sensitive and salt-tolerant clusters.

On the other hand, mature root zone represents a major bulk of the root and, thus, has to deal with the largest quantities of accumulated Na^+. This zone also has fully extended

cells, with large and well-formed vacuoles, while in meritematic or transition zone cells are much smaller, and with small vacuoles (Verbelen et al., 2006). Thus, superior Na^+ sequestration ability in mature root zone make sense from both anatomical and physiological points of view. Another aspect to be considered is a need to maintain cell turgor pressure under hyperosmotic conditions caused by salinity. Indeed, cytosolic Na^+ intensity was *always* higher in elongation zone compared with all other zones in each variety studied; and so was the vacuolar Na^+ intensity (Fig 11). It may be suggested therefore that in this zone Na^+ might be utilized by roots as a cheap osmoticum to maintain turgor pressure and enable cell expansion. Consistent with this idea are findings that in maize root apical zones, the estimated turgor potential showed only a small decline although salt shock caused a rapid decrease in root water and solute potentials in the major bulk of the root (Rodríguez et al., 1997).

Na^+ sequestration into vacuole is believed to be mediated by the tonoplast NHX Na^+/H^+ antiporters (Apse et al., 1999). Indeed, salt tolerant bread wheat variety showed higher expression of *TaNHX* in roots (Saqib et al., 2005b) and also higher vacuolar Na^+ sequestration in root mature zone compared with the sensitive ones (Cuin et al., 2011). Similarly, relative expression of *ZmNHX* increased proportionally to increasing external NaCl concentration in maize inbred line roots (Zörb et al., 2005). Also, transgenic tobacco lines with *TNHXS1* (wheat Na^+/H^+ vacuolar antiporter gene) had greater Na^+/H^+ antiporter activity in root tonoplast vesicles and showed higher salt tolerance than the wild type (Gouiaa et al., 2012). Previous studies in our laboratory highlighted the importance of K^+ retention in mature root zone of various species (Chen et al., 2005, 2007c; Cuin et al., 2008; Smethurst et al., 2008). Here we provide the evidence that the vacuolar Na^+ sequestration in this zone correlates with salinity tolerance. Taken together, cytosolic K^+ retention and Na^+ sequestration represent two major components of the tissue tolerance mechanism. Targeting these traits may be a promising way to improve salinity tolerance in bread wheat.

7.4.2 Root meristem zone as a salt sensor?

How plant sense Na^+ to trigger the following signalling cascades to cope with the salt stress is a fundamental question which still need to be studied and clarified. In animals, Na^+ sensing mechanism appears to consist mostly of specific Na^+ selective ion channels and other Na^+ transporters; however, so far no Na^+ selective ion channels have been identified in plants (Maathuis, 2014). Displacement of Ca^{2+} by Na^+ from the

plasmalemma of root cells was proposed as a primary response to salt stress (Cramer et al., 1985), but it has been deemed to be of a minor importance later (Kinraide, 1999). Plasma-membrane based SOS1 Na^+/H^+ antiporter was also suggested as a potential salt sensor (Zhu, 2003) but the explicit evidence is still lacking. Histidine kinases (such as Hik16/Hik41 in cyanobacterium, Marin et al., 2003; or AHK1/ATHK1 in Arabidopsis, Shinozaki and Yamaguchi-Shinozaki, 2000; Tran et al., 2007) were also named as potential NaCl- and/or osmo-sensors.

Generally, root is the first organ that perceives the salt stress signal. The transition zone in root apex has been suggested as a signalling-response nexus in the root (Baluška et al., 2010). This unique zone provides the root apices with an effective mechanism to reorient growth in response to many stimuli such as salinity, gravity, temperature, moisture, oxygen availability, electric fields, and heavy metals (Verbelen et al., 2006). In our investigation, both cytosolic and vacuolar Na^+ intensity in root transition zone showed no significant difference between salt tolerant and sensitive clusters (Fig. 8C, D); no significant correlation was also found between cytosolic or vacuolar Na^+ intensity in this zone and plant damage index (Suppl. Fig. S4B, S4F). Thus, it appears that root transition zone is *not* the main signalling-response nexus to salt stress, at least in bread wheat.

Salt stress causes nuclear and DNA degradation in root meristematic cells (Katsuhara and Kawasaki, 1996; Liu et al., 2000; Richardson et al., 2001), and NaCl-induced nuclear DNA fragmentation in the root meristem zone was higher in *sos1* mutant (lacking the ability to remove Na^+ to external media) compared with *Arabidopsis* wild type, when grown under saline conditions (Huh et al., 2002). From this point of view, one would expect that salt-tolerant varieties would maintain lower cytosolic Na^+ intensity in meristem cells. This was not the case in our study (Fig. 11). On the contrary, salt tolerant group showed 4.3-fold higher cytosolic Na^+ intensity (159.2 ± 17.9 vs 37.0 ± 8.4, respectively; Fig. 11) than sensitive cluster. At the same time, vacuolar Na^+ intensity was not significantly different between groups (Fig. 7D). It is tempting to suggest that higher cytosolic Na^+ intensity in salt tolerant cluster in the meristematic zone might be important to effectively convey or regulate signals during salt stress to other root zones even shoot after perceiving external salt stress. Also, when plants were subjected to salt stress, the tolerant varieties which had significant higher cytosolic Na^+ might have an advantage to buffering the rapid increase of Na^+ in cytosol buying time for initiating salt stress related adaptive response. Hence, we suggest that, in addition to its role in cell division, root zone

meristem also participates in, or executes, a role of the salt sensor. The specific details of this signalling mechanism should be revealed in further studies.

7.4.3 Evaluating salinity tolerance by screening vacuolar Na^+ sequestration via LSCM technique

In addition to physiological and genetic complexity of the salinity tolerance trait, the progress in breeding was also hampered by the lack of convenient screening techniques. While agronomical (e.g. biomass/yield, plant survival, or leaf injury; Munns and James, 2003; Colmer et al., 2005) and biochemical (e.g. antioxidant activity or compatible solutes content; Ashraf and Harris, 2004) markers are convenient as rapid screening tools, they are not directly linked with major physiological mechanisms conferring salinity tolerance. Thus, the demand for a technique that is, on one hand, convenient enough to be used for the high throughput screening and, on another hand, was directly linked to a specific physiological trait in question, remains high. We believe that using CoroNa Green imaging may suit this purpose.

Na^+ measurement by CoroNa Green dye confocal imaging was firstly conducted in animals (Meier et al., 2006). It was then adopted by researchers to visualize Na^+ distribution in both root (Park et al., 2009; Oh et al., 2009, 2010; Li et al., 2012) and leaf (Bonales-Alatorre et al., 2013a, b) tissues under saline conditions. The present work narrows down salinity tolerance trait to vacuolar Na^+ sequestration in merely one specific root zone (Fig. 10, 11), and showed it high ($r = 0.77$, Suppl. Fig. S4H) prognostic value as a screening tool. In practical terms, imaging of one root for one specific zone takes only few minutes, in addition to ~2 h required for staining. Thus, quantifying Na^+ fluorescent intensity in 100-120 roots per day by one operator is a realistic task. From our experience, 6-8 biological replicates are sufficient to get consistent results and eliminate out layers. Thus, even at the current stage, screening 15 to 20 genotypes per day may be feasible. The next practical step should be creating a DH population between Westonia and Belgrade 3 (varieties with highest and lowest vacuolar Na^+ sequestration ability), and then screen this DH population to determine QTL(s) associated with such sequestration ability. This work is next on agenda in our laboratories.

Another screening approach might arise from the idea to simply measure the total Na^+ concentration in each of the root regions. However, in a typical plant cell the central vacuole occupies up to 90% cell volume. Thus, a possible difference in the cytosolic Na^+ content in the remaining 10% of the cell volume (cytosol) in root meristem will be

undoubtedly masked/buffered by the uncertainty in vacuolar Na^+ content. This is not the issue for mature zone, where the genotypic difference originates from vacuolar Na^+ content. However, the bulk root tissue analysis does not account for the heterogeneity of Na^+ distribution between various types of root cells within the same zone (e.g. those in root stele). Thus, unless the new techniques are developed to sample and analyse the ionic content of the single root cell, the fluorescence method is still most preferred.

Chapter 8

Cell-specific regulation of root ionic homeostasis in the context of salinity stress tolerance: a case study for the elongation zone in barley[#]

Abstract

Cell type specific salinity stress responses in plants have been revealed mostly at the transcriptional level while the functional insights into tissue- and cell- specific regulation of ionic homeostasis in plants remains largely obscure. In this work, we have combined the MIFE technique for non-invasive ion flux measurements with confocal fluorescence dye imaging technique to screen 45 accessions of barley to reveal the essentiality of Na^+ exclusion from the cytosol to apoplast, vacuolar Na^+ sequestration, and cytosolic K^+ retention in the root elongation zone for the overall salinity stress tolerance. We show that SOS1-like Na^+/H^+ antiporter-mediated Na^+ extrusion plays a very little role in the overall salt tolerance in barley. Unlike the mature root zone, cytosolic K^+ retention in the elongation zone did not correlate with genetic variability in salinity tolerance among barley accessions, and salinity-induced K^+ efflux in this zone was not mediated by TEA-sensitive depolarization-activated K^+ channels. At the same time, a strong and positive correlation was found between vacuolar Na^+ sequestration ability and the overall salt tolerance. Salt sensitive genotypes showed significantly higher expression levels of both *HvNHX1* and *HvVP1* compared with tolerant genotypes. It is suggested that the failure of sensitive varieties to sequester Na^+ in root vacuoles despite a pronounced increase in *HvNHX1* and *HvVP1* transcript levels is due to their inability to control Na^+ back-leak into cytosol. Overall, our results demonstrated high tissue specificity of transport processes conferring intracellular K^+/Na^+ homeostasis and its role in the overall salinity stress tolerance in barley.

Keywords: Barley, root elongation zone, salinity stress tolerance, tissue specificity, ion channels/transporters, ion hemostasis

[#], This chapter was submitted as: Wu H, Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S: Plant Physiology.

8.1 Introduction

Soil salinity is a major environmental constraint to crop production that affects about 45 million hectares of irrigated land and costs agriculture an estimated US$ 27.3 billion p.a. in lost revenues (Qadir et al., 2014). Nearly 10% of the land surface and 50% of all irrigated land in the world are affected by salinity (Ruan et al., 2010), and the proportion is still rising (Li et al., 2014). Given the rapid increase in the world population that is expected to exceed 9.3 billion by 2050, identifying salt-tolerant germplasm is essential to ensure the food security in the 21st century.

The major hurdle in achieving this goal lays in the physiological and genetic complexity of the salinity tolerance trait. It has become increasingly evident that different plant cells and tissues respond to salinity stress in a highly specific manner, both genetically (Dinneny et al., 2008; Dinneny, 2010) and physiologically (Nelson et al., 1998; Møller et al., 2009; Plett et al., 2010). This has prompted calls for a targeted gene expression and a need to understand the operation of key salinity–related genes at the cellular level. Gaining this understanding remains a great challenge, largely from the lack of available techniques enabling the functional assessment of the operation of specific transport proteins which mediate plant ionic homeostasis under saline conditions *in planta* at the cellular level with sufficient spatial and temporal resolution. Most papers published in the field still use the whole plant indices such as shoot/leaf Na^+ or K^+ content as proxies for salinity stress tolerance (and hence as a guide in breeding programs), completely ignoring the specifics of both intra and inter-cellular sequestration of these ions.

In most crops, salinity stress tolerance is correlated with the reduced accumulation of Na^+ in the shoot or leaves (Matsushita and Matoh, 1991; Garthwaite et al., 2005; Cuin et al., 2010; Munns et al., 2012). This trait is often erroneously termed as “sodium exclusion”. The main issue with using this terminology is in the fact that the reduced Na^+ accumulation in a shoot could be achieved by at least four different mechanisms: (i) reduced rate of the unidirectional Na^+ uptake by plant roots; (ii) enhanced root Na^+ extrusion back into rhizosphere; (iii) reduced rate of Na^+ loading into the xylem; and (iv) enhanced retrieval of Na^+ from the shoot and its recirculation back to roots. So, which of these (very different) mechanisms makes the biggest contribution to the ’sodium exclusion’ trait?

Previous studies revealed no significant difference in the unidirectional Na^+ influx in roots between salt tolerant and sensitive barley (Chen et al., 2007c) or wheat (Davenport et al., 1997) varieties suggesting that control of Na^+-uptake systems plays a relatively minor role in the overall salinity stress tolerance, at least as it concerns avoiding excessive Na^+ accumulation. So far, SOS1 (salt overly sensitive 1) Na^+/H^+ antiporter is the only transporter which has been shown to mediate Na^+ efflux from the cytosol to apoplast in plant cells. The GUS expression analysis in *Arabidopsis* showed that SOS1 is preferentially expressed in the root apex and vasculature (Shi et al., 2002). Many papers reported increased salt sensitivity in plants lacking functional SOS1 genes (Shi et al., 2000; Olías et al., 2009b), and overexpression of SOS1 Na^+/H^+ antiporter increased salt tolerance in many species e.g. *Arabidopsis* (Shi et al., 2003, Yang et al., 2009), tobacco (Yue et al., 2012), and tomato (Olías et al., 2009b). However, recently Feki et al (2011) showed that overexpressing TdSOS1 in *Arabidopsis* did not result in any significant enhancement of the salt stress tolerance in transgenic plants as compared with the performance of lines transformed with empty vectors. Is this because the wrong cell type was targeted? Indeed, the root apex harbours several functionally different zones (meristem; distal elongation zone; elongation zone) and a broad range of tissues (e.g. epidermis; cortex; stellar tissues). Do they all have the same requirement for the Na^+ extrusion? If not, what determines this need and how is this trait related to other mechanisms conferring intracellular ionic homeostasis, such as vacuolar Na^+ sequestration or K^+ retention?

The best known candidates for the vacuolar Na^+ sequestration are tonoplast NHX1 Na^+/H^+ antiporters (Apse et al., 1999). Although they have been recently shown to be not as selective for Na^+ as originally thought and also mediate substantial fluxes of K^+ across the tonoplast (Bassil et al., 2011), NHX expression levels correlated with higher vacuolar Na^+ sequestration ability (Zörb et al., 2005; Gouiaa et al., 2012), and overexpressing NHX1 resulted in an improved salinity stress tolerance in many species such as *Arabidopsis* (Apse et al., 1999), wheat (Xue et al., 2004), and rice (Chen et al., 2007a). However, in most cases the reported beneficial effects were attributed to improved Na^+ sequestration in shoot tissues (Apse et al., 1999; Brini et al., 2007; Chen et al., 2007a). Also, some papers showed that expressing NHX1 Na^+/H^+ exchanger does not significantly improve the overall salt tolerance (e.g. in *Arabidopsis* - Yang et al., 2009; barley - Adem et al., 2015). Can tissue specificity be a reason for such controversy? And how important is vacuolar Na^+ sequestration in roots in general?

K^+ loss from the plant cell has been recently suggested as a "metabolic switch" which is beneficial for turning a cell into defence mode (Demidchik et al., 2014). At the same time, a root cell's ability to retain K^+ in the cytosol has emerged as a key mechanism in overall salt tolerance in many plant species including barley (Chen et al., 2005, 2007c), wheat (Cuin et al., 2008), and poplar (Sun et al., 2009), with tolerant genotypes showing much less stress-induced K^+ efflux from the root. How can these two (apparently controversial) facts be explained? Is it possible that the role of cytosolic K^+ as a signalling agent (Anschütz et al., 2014) and a "metabolic switch" (Demidchik, 2014) differ in different cell types? It should be noted that in the all above cases that have been reported a positive correlation between NaCl-induced K^+ efflux and sensitivity to salt stress, measurements were conducted in the mature root zone. Can these observations be extended to other cell types in the root?

The root apex is arguably the most metabolically active and dynamic tissue in a plant (Verbelen et al., 2006; Baluška et al., 2010). Cells in the root elongation zone quadruple their size in only two hours (Verbelen et al., 2006); this implies a need for massive fluxes of ions and water to exert turgor pressure and expand the cell. It was also shown that elongating root cells are the primary targets for a range of abiotic stresses such as Al (Ma et al., 2004), low pH (Koyama et al., 2001) and heavy metal (Potters et al., 2007) toxicity. They also possessed higher ROS sensitivity compared with cells in the mature root zone (Demidchik et al., 2007). Interestingly, the highest Na^+ accumulation was found in both cytosolic and vacuolar compartments in the root elongation zone in bread wheat compared with any other cell types (measured as CoroNa Green fluorescence intensity; Wu et al., 2015a). Moreover, tolerant bread wheat varieties had 20% higher fluorescent signal compared with sensitive varieties (Wu et al., 2015a). This raises a question: to what extent is the ability for Na^+ extrusion linked with the overall salt tolerance in plants? Is this a "rule of thumb" trait, or could it be that cytosolic Na^+ may operate as a signal triggering gene expression and regulating a complex network of signals aiming to tune up plant metabolism for altered conditions in some cell types? If this is the case, what other traits are essential to avoid detrimental effects of elevated Na^+ in the cell cytosol?

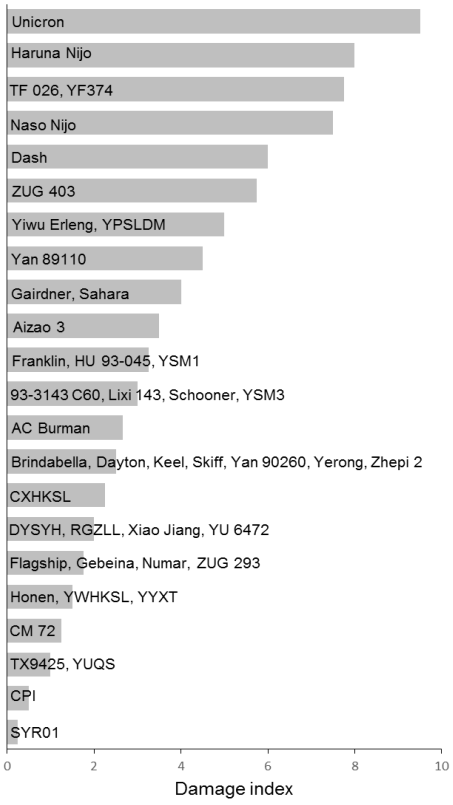
As commented earlier, the lack of answers to these and other questions come from the lack of available techniques enabling the functional assessment of the operation of specific transport proteins *in planta* at the cellular level. In this work, we attempted to bridge this gap by applying two cutting edge tools - the non-invasive microelectrode ion flux estimation (the MIFE) technique and CoroNa Green fluorescence dye imaging – to

study kinetics of Na^+ and K^+ movement across cellular membranes and their intracellular sequestrations in various compartments in the elongation root zone of a large number (45 in total) of barley varieties contrasting in their salinity stress tolerance. By doing this, we have assessed the essentiality of three key traits for determining cellular ionic homeostasis - namely Na^+ extrusion from the cytosol, vacuolar Na^+ sequestration, and K^+ retention in the cytosol - in salinized barley root elongation zone and determined their relative contribution towards the overall salinity stress tolerance in this species. We have also evaluated and compared the importance of transcriptional and post-translational regulation of appropriate genes conferring these traits. The reported data is discussed in the context of complexity of tissue and cell-specific plant responses to salinity.

8.2 Materials and methods

8.2.1 Plant materials and growth conditions

Forty-three common barley (*Hordeum vulgare*) and two wild barley (*H vulgare* ssp. *Spontaneum*, SYR01 and CPI 11284-48) genotypes contrasting in their salinity stress tolerance (Suppl. Fig. S1) were used in this study. Seeds were obtained from the multiple sources and multiplied in our laboratory. Sterilized seeds were grown hydroponically in 1 L containers in the double distilled water (ddH_2O) for three days at 25±1°C temperature in the dark before salinity treatment (100 mM NaCl for 24 h) was given.



Suppl. Fig. 1. Genetic variability of overall salt tolerance in 45 barley varieties. Damage index of 45 barley varieties was showed. Damage index was ranged from the lowest 0.25 (SYR01) to the highest 9.5 (Unicorn).

8.2.2 Glasshouse experiments

Twelve to 14 seeds of each genotype were sown in a 4.5 L PVC pot filled with standard potting mix (see Chen et al. 2007d for all details) and grown in Jan-Feb 2013 and 2014 under glasshouse facilities at the University of Tasmania. Once established, plants were thinned to leave eight uniform seedlings in each pot. Plants were irrigated twice per day by an automatic watering system with dripper outlets for both control condition and salinity treatment. A saucer was placed under each pot. Salinity treatment (300 mM NaCl in the irrigation solution) was started when seedlings were five days old and lasted for 40 days. Experiments were conducted in triplicate and repeated over two consecutive seasons. The damage index of each variety was scored on a 1-10 scale based on its performance under salinity treatment, with higher damage index numbers representing the lower tolerance (Suppl. Fig. S1). The full details of plant scoring for damage index are available in our previous publications (Wu et al. 2015d).

8.2.3 Preparation of ion selective microelectrodes for non-invasive ion flux measurements

Net ion fluxes (Na^+, K^+ and H^+) were measured non-invasively using the MIFE (microelectrode ion flux estimation) technique (Shabala et al., 2006). Briefly, borosilicate glass capillaries (GC150-10; Clark Electrochemical instruments, Pangbourne, Berks, UK) were pulled using a vertical puller. The pulled electrodes were then dried in an oven at 225ºC overnight, and silanized with tributilchlorosilane (Cat. No 90796; Fluka, Busch, Switzerland). After drying and cooling, electrodes were back filled with backfilling solutions (for H^+ - 15 mM NaCl + 40 mM KH_2PO_4, pH 6.0 adjusted using NaOH; for Na^+ - 500 mM NaCl; for K^+ - 200 mM KCl) and tips of respective electrodes were front filled with commercially available selectophore cocktails (K^+, Cat. No 60031, Sigma; H^+, Cat. No 95297, Sigma-Aldrich, St, Louis, MO). An improved calixarene-based Na^+ ionophore cocktail was used for Na^+ measurement (for details, please refer to Jayakannan et al., 2011). Only electrodes with a slope above 50 mV per decade and correlation 0.999 or higher were used.

8.2.4 Functional assessment of the SOS1-like activity in barley roots by the MIFE technique

The functional assessment of the SOS1-like activity in barley root apex was conducted using the protocol developed and validated by Cuin et al. (2011). A set of

pharmacological experiments and SOS1 gene expression assay were done to confirm that the active Na extrusion from roots under Na^+ recovery protocol is mediated by SOS1-like plasma membrane transporter, at least in wheat (Cuin et al., 2011). After removal of the applied salt, the Na^+ efflux measured from roots in the non-ionic (sorbitol) solution isotonic to the applied salt is very similar to the reported "Na^+ recovery protocol" one (Cuin et al., 2011). In brief, 4-d old hydroponically-grown plants treated with 100 mM NaCl for 24 h were gently rinsed with 10 mM $CaCl_2$ for 1 min to remove all the apoplastic Na^+. Seedlings were placed in a Petri dish containing ddH_2O. 3cm long root segments were then cut starting from the root apex. Excised root segments were immediately immobilized in the Perspex holder and placed in a chamber containing ddH_2O and left for 30 min. Na^+ and H^+ electrodes were positioned ~40 μm above the root surface in the elongation zone (1 to 2 mm from the tip) under x100 microscopic magnification. During measurements, electrodes were moved by a computer-controlled hydraulic manipulator between two position (40 and 110 μm from the root surface), in a 12-s square-wave cycle. Steady state Na^+ flux and H^+ flux were recorded and displayed on a screen. Fluxes were measured for 5 minutes. The recorded voltage outputs of the electrodes were converted into net fluxes by using cylindrical diffusion geometry (Newman 2001) and expressed in $nmol^{-2}\ s^{-1}$ using MIFEFLUX software (Newman 2001; Shabala et al. 2006).

8.2.5 Quantifying K^+ retention in barley root cells

The ability of epidermal root cells to retain K^+ was quantified using the protocols developed and validated in our previous studies (Chen et al. 2005, 2007c). Root segments were isolated from 4-d old plants grown hydroponically in ddH_2O. Excised segments were mounted in a chamber with a Perspex holder and incubated in BSM (Basic Salt Media; 0.5 mM KCl + 0.1 mM $CaCl_2$) with 100 mM NaCl added. Ion-selective microelectrodes were positioned next to the root as described above, and steady net fluxes of K^+ and H^+ were measured from the epidermal cells in the elongation zone (1 to 2 mm from the tip) after 1h of 100 mM NaCl treatment, when steady-state fluxes were reached (Chen et al., 2007d).

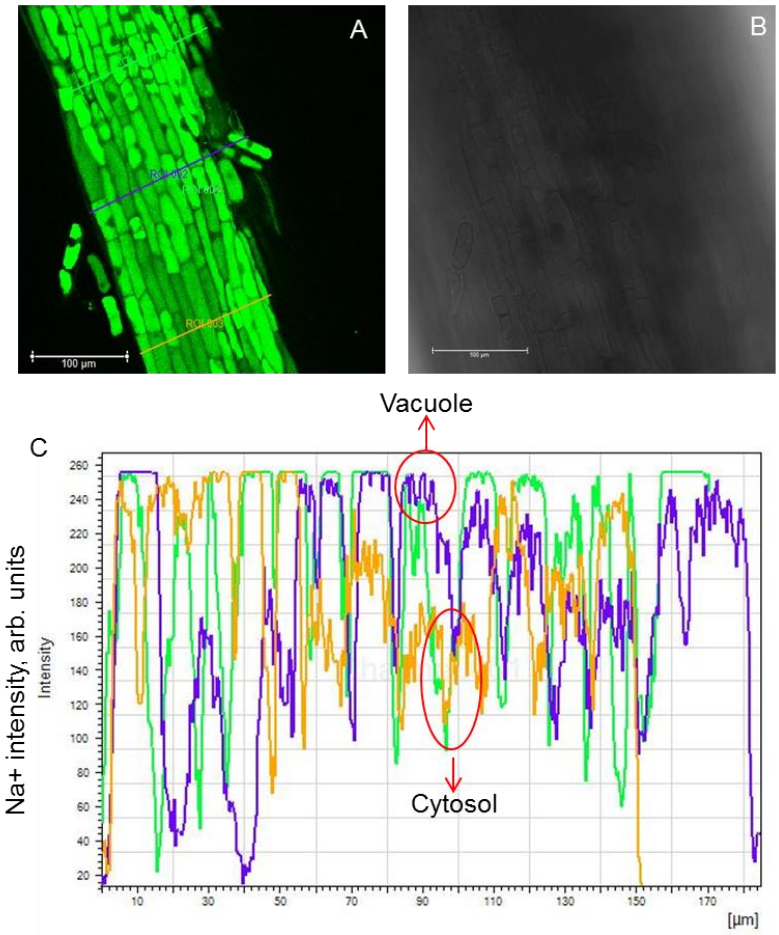
8.2.6 Quantifying intracellular Na^+ distribution by the laser confocal scanning microscopy (LCSM)

Na^+ intensities in the cytosolic and vacuolar compartments in barley root cells were measured by fluorescent CoroNa Green acetoxymethyl ester Na^+ dye using protocols developed and validated in our previous publications (Wu et al., 2015a). Briefly, root apex segments were cut at 10 mm from the tip, and incubated for 2h in the dark in a solution containing 20 μM CoroNa Green. Root segments were then rinsed in a buffered MES solution and examined using an upright Leica Laser Scanning Confocal Microscope SP5 (Leica Microsystems, Germany) equipped with a 40× oil immersion objective (excitation wavelength 488 nm; emission 510-520 nm). LAS Lite software was used to analyse the confocal microscopy results. As shown in the Suppl. Fig. S2, three lines of "ROI" (Region of Interest) were drawn across root elongation zone cells and then plotted as continuous fluorescence intensity distribution profiles in an Excel file (quantified in arbitrary units by LAS software). Mean cytosolic and vacuolar Na^+ fluorescence intensities were then calculated for each cell by attributing signal profiles to the root morphology (visualised by the light microscopy images) and averaged data from all measured cells for the same treatment were recorded for further analysis. Six to eight individual roots (the longest root from each plant) from each genotype were used during analysis; and at least 2 (two) images were taken from each root apex. The background signal was measured from the empty region and then subtracted from the readings, to obtain corrected fluorescence values. Comparison of the fluorescence signals in the cell cytosol and vacuole was done under the identical imaging settings of the confocal microscope (i.e. exposure times, laser intensity, pinhole diameter and settings of the imaging detectors). Altogether, readings from between 60 to 100 cells were averaged and reported for each genotype. The CoroNa Green Na^+ fluorescence intensity in roots grown under Na^+-free condition was very marginal (Wu et al., 2015a) and not reported here.

8.2.7 Pharmacological experiments

The identity of Na^+ and K^+ transport systems underlying NaCl-induced ion fluxes across barley root cell membranes was investigated in a series of pharmacological experiments using some known channel blockers and metabolic inhibitors. One representative variety (cv Gebeina) was used in all experiments. For Na^+ flux experiments, excised, washed and immobilized roots from salt-grown plants (see above) were pre-treated for 1h with 0.1 mM amiloride (a known inhibitor of Na^+/H^+ exchange activity in both animal (Kleyman and Gragoe, 1988) and plant (Cuin et al., 2011) cells) before starting MIFE measurements. For K^+ flux experiments, excised roots of control-

grown plants were pre-treated for 1h with one of: 20 mM tetraethyl ammonium chloride (TEA^+, a known blocker of K^+ selective channels), 0.1 mM gadolinium chloride (Gd^{3+}, a known blocker of NSCC channels), and 1 mM vanadate (a known inhibitor of H^+-ATPase) in the presence of 100 mM NaCl, before starting MIFE measurements. Net steady state Na^+ and K^+ fluxes were then recorded for 5 min. All chemicals used were from Sigma-Aldrich, St, Louis, MO.



Suppl. Fig. 2. Illustration of the quantification procedure for Na^+ distribution between the cytosol and the vacuole. Several lines (transects?) are drawn across the so-called "region of interest" (ROI) in elongating root cells in barley variety YUQS (A). Corresponding light images are shown in B. Continuous fluorescence intensity distribution profiles (C) are obtained by LAS software. The mean fluorescence intensity values for cytosolic and vacuolar compartments are then calculated for each cell by attributing signal profiles to root morphology (visualised by light microscopy images). For details, please refer to Wu et al., 2015a. Note: All of the confocal imaging measurements were taken under the same conditions, from what we screened in this thesis (about 50 wheat and 50 barley varieties), the elongation zone always showed the highest Na^+ intensity.

8.2.8 Quantitative real-time PCR analysis

The root apexes (~3 mm from the root tips) of 4-d old hydroponically grown barley seedlings treated with 100 mM NaCl for 24 h were harvested and snap frozen in liquid nitrogen. Two varieties contrasting in salinity stress tolerance (Gebeina - salt tolerant, and

Unicorn - salt sensitive, Suppl. Fig. S1) were used. The root RNA was extracted by using the RNeasy Mini Kit (QIAGEN) followed by the manufacture's instruction (Note: The genomic DNA was wiped out by using the QIAGEN RNeasy Mini Kit). cDNA was synthesised by using QuantiTect® Reverse Transciption Kit (QIAGEN) followed by the manufacture's instruction. Quantitative real-time PCR was performed as described in Vandesompele et al. (2002) by using a RG6000 Rotor-Gene Q Real Time Thermal Cycler and SYBR green PCR reagent (QIAGEN). The relative expression level of studied genes was analysed by using $2^{-\Delta\Delta C_T}$ methods (Livak and Schmittgen 2001). Primers were designed to determine the expression of the transporters *HvSOS1* (MLOC_36509.1, PGSB), *HvNHX1* (AK376115, PGSB), and the channel *HvGork* (AK376785). Primer sequences are given in Suppl. Table S1. Normalization of the test gene transcript was relative to the control gene (*GAPdH2*). Experiments were repeated three times, with consistent results.

Target genes	**Locus**	**Primer sequences**
Hv-SOS1	MLOC_36509.1	F: 5'-AAGCACAGCAAGTCGTATCC-3'
		R: 5'-GAGTTGTCATCTTCCGCTATCT-3'
Hv-VP1	AY255181.1	F: 5'-GTCTCGGCGGTTCTTCCAT-3'
		R: 5'-CAACAACAAGAGCAGCACAAG-3'
Hv-NHX1	AK376115	F: 5'-TGCATATCTACCAGTGCTTAT-3'
		R: 5'-GGTTCAAGACACAAGTTCAGT-3'
Hv-GORK	AK376785	F: 5'-CCACACGAGGCGAAGAAG-3'
		R: 5'-GAGGAATCCACAGCATCACC-3'
Hv-GAPdH2	KC661222.1	F: 5'-GTGAGGCTGGTGCTGATTACG-3'
		R: 5'-TGGTGCAGCTAGCATTTGAGAC-3'

Suppl. Table 1 – Primers used for quantitative real-time PCR.

8.2.9 Statistical analysis

All data (given as mean ± SE, n = sample size) were analysed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). All of the replicates are biological replicates. Comparison of Na^+ and H^+ fluxes in barley root elongation zone after removal of external salt between control and amiloride treatment was done by independent samples *t*-test. The significance levels are $^{**}P < 0.01$ and $^{***}P < 0.001$. The significance of the correlation between different parameters was determined by Bivariate Correlations based on Pearson Correlation (2-tailed). The data used for correlation analysis are the average values of measured independent parameters for each variety. The significance within the effects of different pharmacological treatments and different clusters was determined by one-way

ANOVA based on Duncan's multiple range test. Data labelled with different lower case letters is significant at $P < 0.05$.

8.3 Results

8.3.1 SOS1-mediated Na^+ extrusion from the root elongation zone does not correlate with salt tolerance in barley

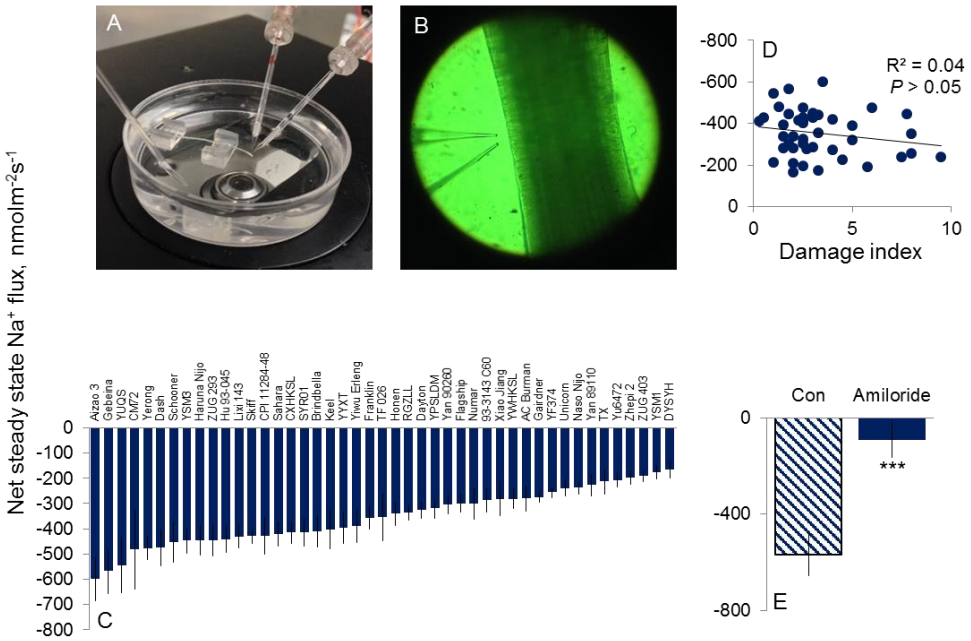


Fig. 1. Genetic variability of SOS1-like antiporter mediated Na^+ efflux in barley root elongation zone and its correlation with overall salt tolerance. (A, B) Representative images of net steady state Na^+ efflux measured by using MIFE technique. (C) Net steady state Na^+ efflux in root elongation zone in 45 barley cultivars. Mean ± SE (n= 6−8). (D) Correlation between net Na^+ efflux in the root elongation zone and damage index. Each point represents a separate variety. (E) Net steady state Na^+ efflux in the root elongation zone (variety Gebeina) treated with amiloride. Mean ± SE (n= 6−8). *** means $P < 0.001$.

When roots of salt-grown plants were transferred into the Na-free solution a massive Na^+ efflux was measured (Fig 1C) reflecting the fact that roots accumulated substantial amounts of Na^+ and had their Na^+ extrusion systems activated. The measured Na^+ efflux was highly sensitive to amiloride, a known inhibitor of Na^+/H^+ exchanger (84% suppression; $P < 0.01$; Fig. 1E), suggesting that the major contributor to this efflux was SOS1-like Na^+/H^+ exchanger at the root plasma membrane. Net Na^+ efflux showed 3.6-fold variability amongst 45 varieties measured, ranging from the highest -600 ± 84 (Aizao3) to the lowest -166 ± 33 (DYSYH) nmol m^2s^{-1} (Fig. 1C). No significant correlation was found between Na^+ extrusion and the overall salt tolerance ($r = 0.20$, $P > 0.05$; Fig. 1D). The above findings were further corroborated by H^+ flux data (Fig. 2). Net

H^+ uptake also showed strong amiloride sensitivity (Fig. 2B), and a significant positive correlation was found between net Na^+ efflux and H^+ influx in barley root elongation zone ($r = 0.36$, $P < 0.05$; Fig. 2C). Taken together these results strongly suggest that in barley Na^+ extrusion from root elongation zone is mediated by the SOS1-like Na^+/H^+ antiporter but the function of SOS1 gene does not determine the genetic variability in the overall salinity stress tolerance.

A further support for the above statement came from cluster analysis of Na^+ efflux (Fig 3). Based on root Na^+ extrusion ability and the overall salt tolerance of plants, five clusters were identified (Fig. 3A). Plants in clusters 1 to 4 showed a high variability in their SOS1-mediated Na^+ efflux ability but yet possessed an overall similar salinity stress tolerance at the whole-plant level. Clusters 1 and 4 had a 3-fold difference in the root Na^+ extrusion ability ($P < 0.01$; Fig. 3C) but similar salt tolerance (damage index; Fig. 3B). At the same time, plants in clusters 3 and 5 possessed the same SOS1 functional activity (Fig. 3C) but were dramatically different in the overall salinity stress tolerance at the whole-plant level (Fig. 3B). Cluster 5 included all sensitive barley varieties tested: Naso Nijo, Haruna Nijo, TF026, YF374, and Unicorn. Thus, it appeared that genetic variability in root SOS1 Na^+ efflux ability was not sufficient to account for the differential salinity stress tolerance, and that other traits such as K^+ retention and/or vacuolar Na^+ sequestration might play a bigger role(s) in the overall plant performance under saline conditions.

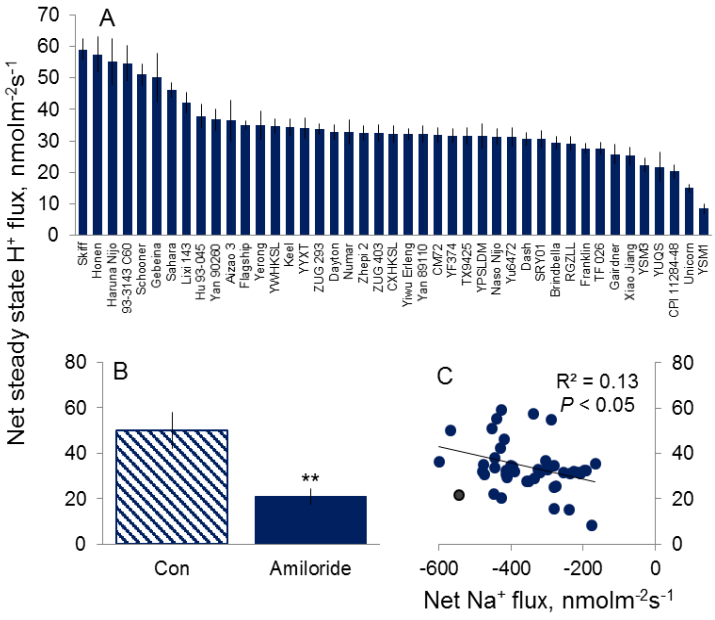


Fig. 2. Genetic variability of SOS1-like antiporter mediated H^+ influx in barley root elongation zone and its correlation with overall salt tolerance (A) Net steady state H^+ influx in root elongation zone in 45 barley cultivars. Mean ± SE (n= 6−8). (B) Net steady state H^+ influx in root elongation zone (variety Gebeina) treated with amiloride. Mean ± SE (n= 6−8). ** means $P < 0.01$. (C) Correlation between net H^+ influx in root elongation zone and damage index. Each point represents a separate variety.

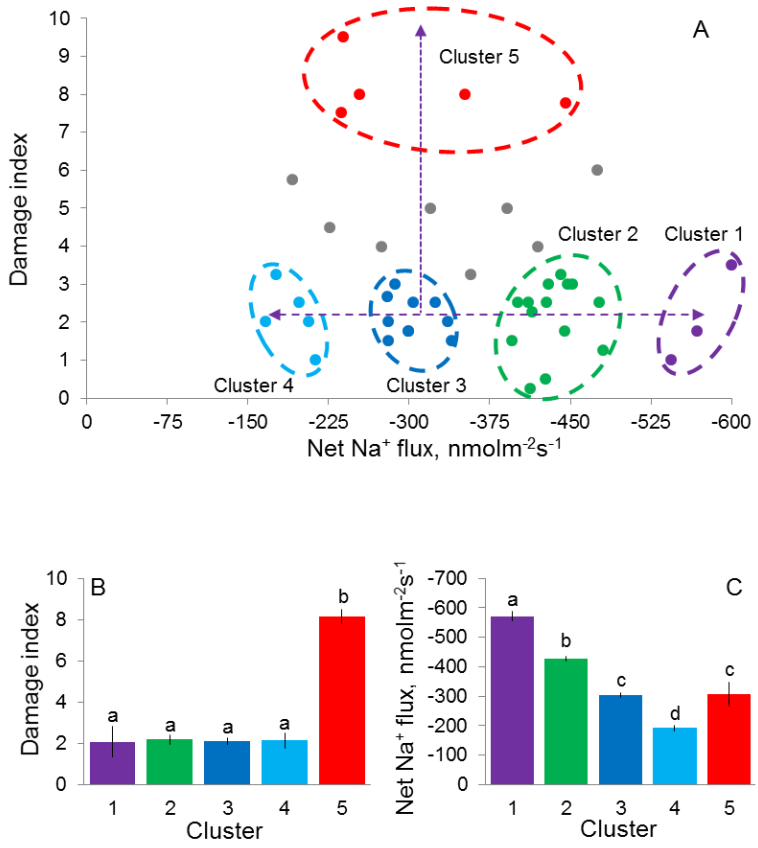


Fig. 3. 45 barley varieties were clustered according to its root Na^+ exclusion ability and overall salt tolerance. (A) 45 barley varieties were clustered into five clusters according to its root Na^+ exclusion ability and overall salt tolerance. (B) Comparison of overall salt tolerance in five clusters. (C) Comparison of Na^+ exclusion ability in root elongation zone in five clusters. Mean ± SE (n= 3−14). Different lowercase letters represent significant difference between clusters ($P < 0.05$).

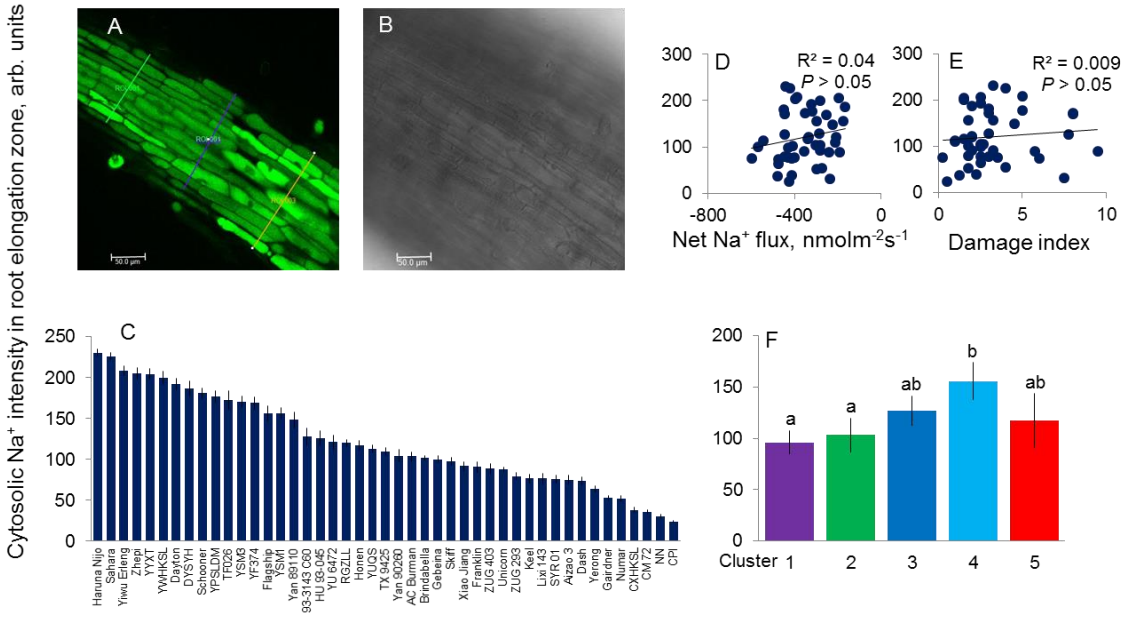


Fig. 4. Genetic variability of Na^+ intensity in cytosol in elongation root cell. Several lines are drawn across the so-called "region of interest" (ROI) in CoroNa Green fluorescence image of elongation root cell in salt tolerant barley variety Gebeina (A) and the corresponding light images (B). (C) Genetic variability of cytosolic Na^+ intensity in root elongation zone of 45 barley varieties. Mean ± SE (n= 72 - 96 cells from at least 6 individual plants). Correlation between cytosolic Na^+ intensity in elongation root cell and Na^+ exclusion (D) and damage index (E). (F) Comparison of cytosolic Na^+ intensity in root elongation zone in selected five clusters. Mean ± SE (n= 3−14). Each point represents a separate variety. Different lowercase letters represent significant difference between clusters ($P < 0.05$).

8.3.2 Vacuolar Na^+ sequestration in the root elongation zone is essential for the overall salt tolerance

Intracellular Na^+ distribution and genetic variability in the vacuolar Na^+ sequestration ability was assessed by Confocal Laser Scanning Microscopy using fluorescent CoroNa Green dye (Figs 4 & 5). Fluorescent signals from the cytosol varied almost 10-fold between varieties, ranging from the highest 230 ± 4.5 (Haruna Nijo) to the lowest 24 ± 1.0 (CPI 11284-48) (Fig. 4C). Consistent with the MIFE Na^+ flux data, the highest cytosolic Na^+ concentration was found in plants of Cluster 4 (Fig. 4F) e.g. ones showing smallest Na^+ efflux (Fig. 3C). However, no significant correlation was found between cytosolic Na^+ signal and either root Na^+ extrusion in the elongation zone ($r = 0.20$, $P > 0.05$; Fig. 4D) or the overall salinity tolerance ($r = 0.09$, $P > 0.05$; Fig. 4E).

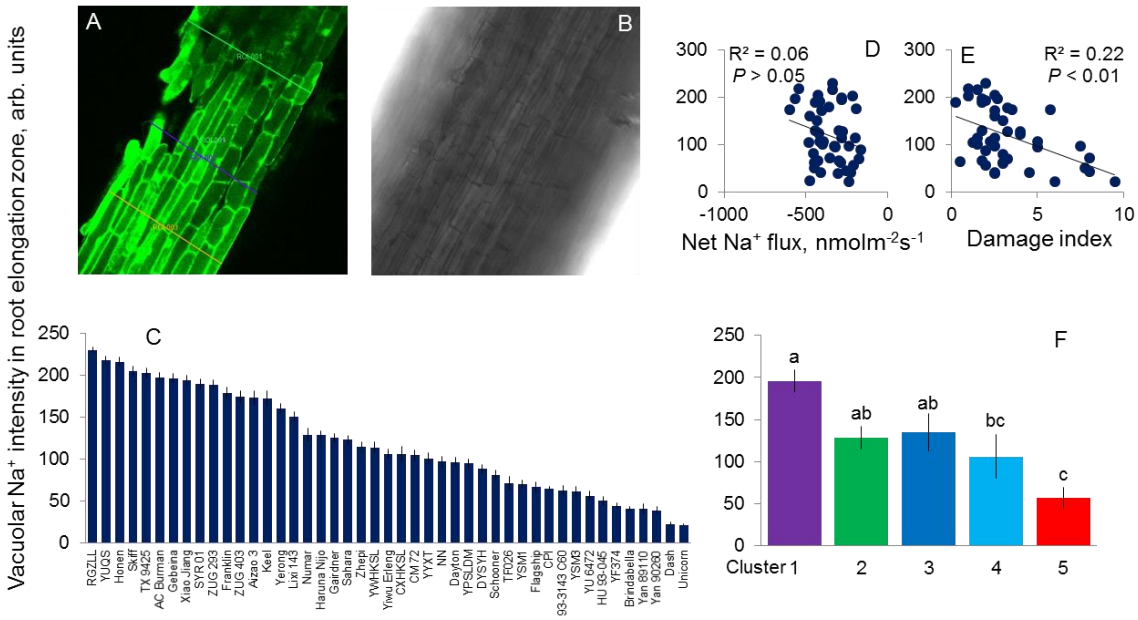


Fig. 5. Genetic variability of Na^+ intensity in vacuole in elongation root cell. Several lines are drawn across the so-called "region of interest" (ROI) in CoroNa Green fluorescence image of elongation root cell in salt sensitive barley variety Unicorn (A) and corresponding light images (B). (C) Genetic variability of vacuolar Na^+ intensity in root elongation zone in 45 barley varieties. Mean ± SE (n= 72 - 96 cells from at least 6 individual plants). Correlation between vacuolar Na^+ intensity in elongation root cell and Na^+ exclusion (D) and damage index (E). (F) Comparison of vacuolar Na^+ intensity in root elongation zone in selected five clusters. Mean ± SE (n= 3−14). Each point represents a separate variety. Different lowercase letters represent significant difference between clusters ($P < 0.05$).

Broad genetic variability (10.6-fold) was also reported for the vacuolar Na^+ intensity, with signals ranging from the highest (230 ± 4.0 in variety RGZLL) to the lowest (21.6 ± 1.0 in variety Unicorn) (Fig. 5C). Vacuolar Na^+ intensity showed significant positive

correlation (r = 0.47, $P < 0.01$; Fig. 5E) with the overall salt tolerance, with varieties possessing superior vacuolar Na^+ sequestration showing lesser extent of salinity damage. Correlation between vacuolar Na^+ content and SOS1 Na^+ efflux ability was not significant (r = 0.25, $P < 0.05$; Fig. 5D). Unexpectedly, the highest ($P < 0.01$) vacuolar Na^+ intensity was found in Cluster 1 which also possessed highest Na^+ efflux ability (Fig. 3C), and was poorest for plants in Cluster 5 (Fig. 5F) that were deemed to be most salt-sensitive (Fig. 3B).

8.3.3 K^+ retention in the root elongation zone does not correlate with salt tolerance and is not controlled by TEA-sensitive voltage-gated channels

Previous studies have revealed that 65% of genetic variability in salinity tolerance in barley is conferred by its ability to retain K^+ in the mature root zone (Chen et al., 2007b) and showed a critical role of depolarization-activated outward-rectifying K^+ efflux channels as a major path for NaCl-induced K^+ leak in this tissue (Chen et al., 2007b). Here we have asked if K^+ retention in the elongation zone also plays such an important role in the overall salinity stress tolerance, and how is it achieved. For doing this, steady state net K^+ fluxes were measured from the root elongation zone using the MIFE technique in salinized barley roots. A 5.9-fold variability in the K^+ retention ability was found amongst 45 barley varieties, ranging from the highest -1190 ± 219 (Skiff) to the lowest -202 ± 27 (DYSYH) nmol m^2s^{-1} (Fig. 6A). To our surprise, no significant correlation was found between K^+ retention in the root elongation zone and the overall salt tolerance in 45 barley varieties (r = 0.08, $P > 0.05$; Fig. 6D). Yet, cluster analysis has revealed some interesting trends. Plants in cluster 4 that had poorest Na^+ extrusion ability (Fig. 3C) showed the highest K^+ retention ability (Fig. 6C), suggesting a compensatory mechanism. Another interesting observation was the lack of any significant ($P < 0.05$) effects of either TEA (a known blocker of K^+-selective channels; Ache et al., 2000) or vanadate (a known blocker of H^+-ATPase and thus a potential modulator of voltage-gated K^+ channels) on NaCl-induced K^+ fluxes (Fig. 6A). At the same time treatment with Gd^{3+} (a known blocker of K^+ permeable non-selective cation channels, NSCC) resulted in ~50% inhibition of NaCl-induced K^+ efflux in the root elongation zone (Fig. 6B, Fig. 7B; $P < 0.05$). At the same time, in the mature root zone NaCl-induced K^+ efflux was strongly (~ 80% suppression) blocked by TEA pre-treatment (Fig. 7A). Taken together, these results indicate that K^+ retention in the elongation root zone plays a smaller role in the overall salinity stress tolerance compared with retention in mature root cells, and that

NaCl-induced K^+ efflux from these tissues is controlled by different molecular mechanisms.

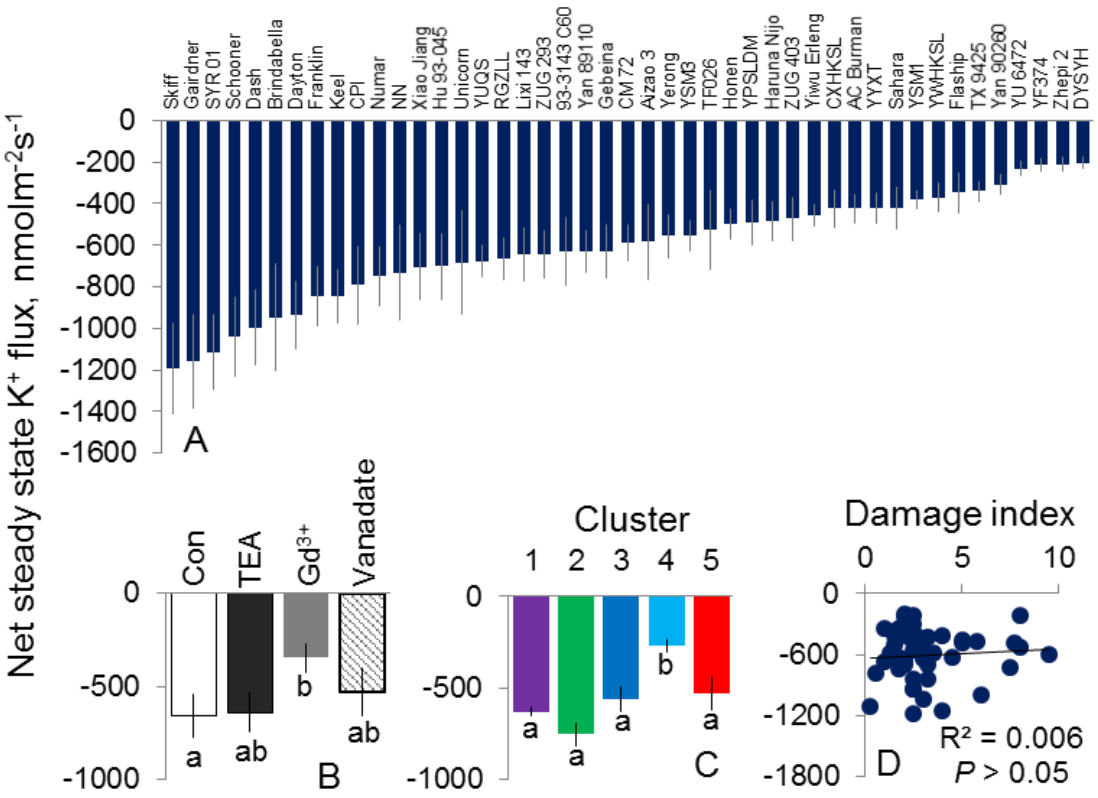


Fig. 6. Genetic variability of NSCC channel mediated NaCl-induced K^+ efflux in the root elongation zone and its correlation with overall salt tolerance. (A) Net steady state NaCl-induced K^+ efflux in the root elongation zone in 45 barley cultivars. Mean ± SE (n= 6−8). (B) Net steady state NaCl-induced K^+ efflux in the root elongation zone (variety Gebeina) treated with TEA, Gd^{3+}, and vanadate. Mean ± SE (n= 6−8). (C) Comparison of K^+ retention ability in the root elongation zone in selected five clusters. Mean ± SE (n= 3−14). (D) Correlation between NaCl-induced K^+ efflux in the root elongation zone and damage index. Each point represents a separate variety. Different lowercase letters represent significant difference between treatments or clusters (P < 0.05).

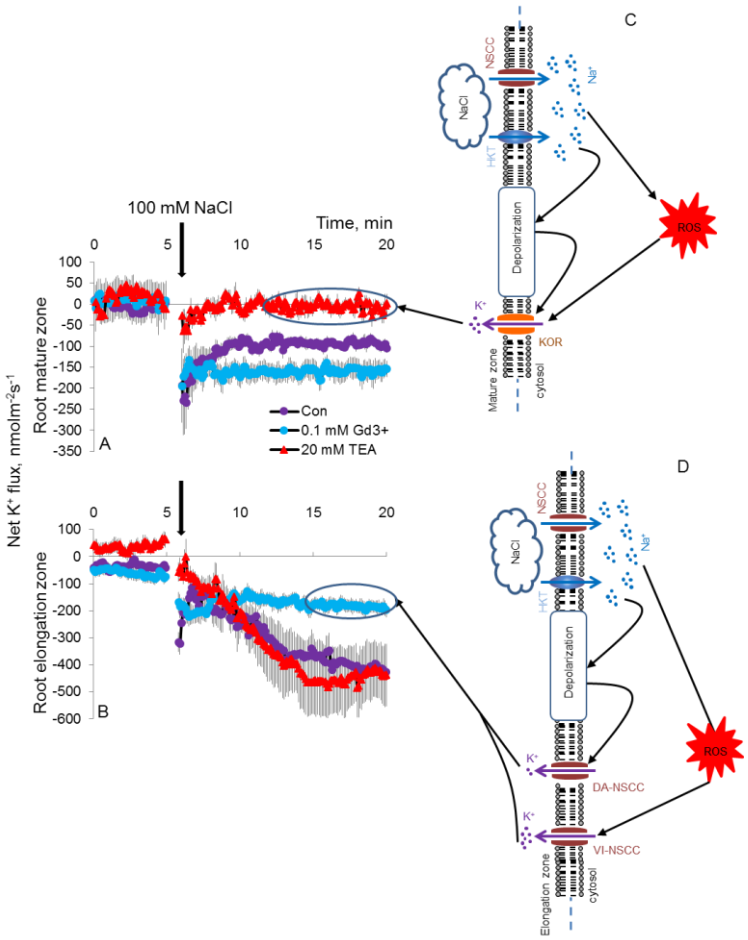


Fig. 7. Channel specificity of mediating NaCl-induced K^+ efflux between root mature and elongation zone (10 and 1-2 mm from the root tip respectively). K^+ flux response to 100 mM NaCl in root mature (A) and elongation (B) zone in plants (variety CM72) under control and 0.5 h pretreatment with TEA and Gd^{3+}. NaCl-induced K^+ efflux was regulated by different channels between root mature (C) and elongation (D) zone. Mean ± SE (n= 6−8).

8.3.4 Relative expression level of *HvSOS1*, *HvNHX1*, *HvGork*, and *HvVP1* in salinized barley root apex

24 h of 100 mM NaCl treatment caused about 60% increase in *HvSOS1* transcript levels in the root apex in both salt tolerant (Gebeina) and salt sensitive (Unicorn) barley varieties although this increase was not statistically significant at $P < 0.05$ (Fig. 8A) and could hardly explain a 3.6-fold variability in the net Na^+ efflux amongst varieties measured (Fig 2A). Combined with the functional assessment of the SOS1 activity by the MIFE technique these results indicate that neither transcriptional nor post-translational regulation of *HvSOS1* determine the overall salinity stress tolerance in barley. Transcripts of *HvGork* increased dramatically by ~20-folds under salt stress (Fig. 8B), with higher expression of the *HvGork* gene reported for salt sensitive genotype. Salinity stress also resulted in a massive 20-fold increase in expression of *HvNHX1* gene but only in a sensitive variety (Fig. 8C). Also, transcripts levels of H^+-PPase (*HvVP1*) increased under saline conditions, and only in the sensitive variety (Fig. 8D).

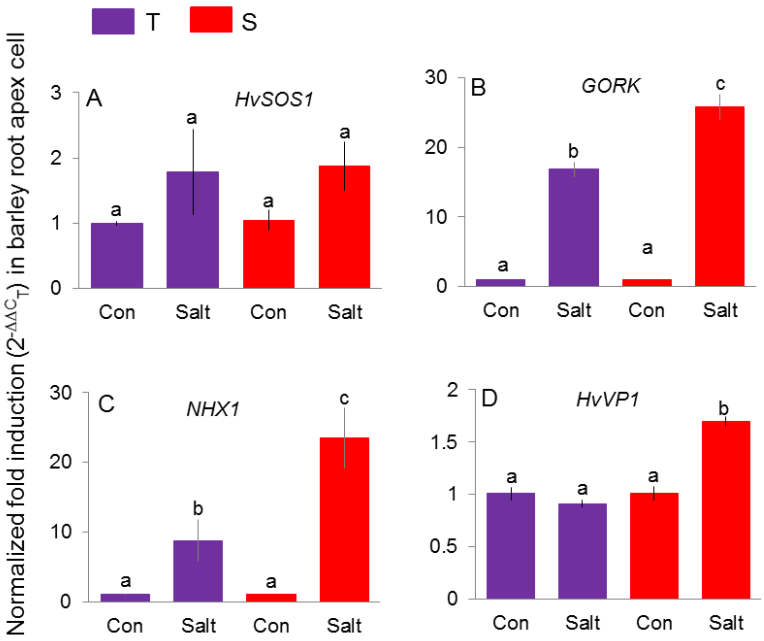


Fig. 8. Relative expression level of *HvSOS1*, *HvNHX1*, *HvGORK*, and *HvVP1* in salinized barley root apex. Relative expression level of *HvSOS1* (A), *HvNHX1* (B), *HvGORK* (C), and *HvVP1* (D) in salinized root apex was studied in barley variety Gebeina (salt tolerant) and Unicorn (salt sensitive) under control and salt stress (100 mM NaCl, 24h). Mean ± SE (n = 8–9) from three replicates. Different lowercase letters represent significant difference at $P < 0.05$.

8.4 Discussion

Until now, most of our knowledge about cell-type specific mechanisms conferring salinity stress tolerance has come from transcriptional studies (Claes et al., 1990; Löw et al., 1996; Kreps et al., 2002; Dinneny et al., 2008; Dinneny, 2010). Here we provide the first large-scale comprehensive *functional* assessment of the activity of several key transport systems mediating intracellular ionic homeostasis in plant cells using a wide range of barley genotypes with contrasting salinity stress tolerance. We show that, in a contrast to reports obtained at the whole plant or shoot/leaf level (Chen et al., 2005; James et al., 2006; Rivandi et al., 2011), Na^+ efflux from the cytosol in the root elongation zone plays very little role in the overall salinity stress tolerance, at least in these varieties. We also show that essentiality for the cytosolic K^+ retention differ drastically between elongating and mature root cells and is controlled by very different types of plasma membrane transporters. Furthermore, we provide the evidence that an increase in the transcripts level of NHX1 tonoplast exchangers and vacuolar H^+-PPase by itself is not

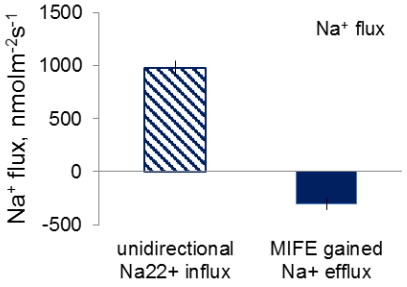
sufficient to achieve efficient vacuolar Na^+ sequestration in the root elongation zone under salt stress.

8.4.1 Na^+ extrusion from root uptake is not a critical factor conferring Na^+ accumulation in the shoot

The general consensus repeatedly stated in the multiple reviews (Tester and Davenport, 2003; Maathuis et al., 2014) is that Na^+ exclusion is a hallmark of salinity stress tolerance in glycophytes. However, as commented in the introduction, there is a tendency to use the term "sodium exclusion" as a synonym for the reduced Na^+ accumulation in a shoot. Here we show that SOS1- mediated Na^+ extrusion from the root does not correlate with the overall salt tolerance, at least in barley (Fig. 1E). At least two possible explanations can be put forward. First, one can speculate that Na^+ accumulation in the root tissues is not as harmful for plants as in the shoots. Several lines of evidence support this notion. First, as reported in Wu et al (2015a; Chapter 7), salt tolerant wheat varieties accumulated larger amounts of Na^+ in the root apex compared with their salt-sensitive counterparts. Second, near-isogenic lines were developed containing either the *Nax1* or *Nax2* loci conferring *TmHKT1;4* (Huang et al., 2006) and *TmHKT1;5* (Byrt et al., 2007) Na^+ transporter of the *HKT* gene family, respectively, had lower rates of Na^+ transport from roots to shoots and higher overall salinity stress tolerance (James et al., 2006b). When *Nax* loci was introgressed into an elite Australian durum cultivar, a 25% yield increase was reported (Munns et al. 2012). However, physiologically these HKT family transporters operated in retrieving Na^+ from the xylem vessels thus restricting the transport of Na^+ from the root to the leaves (Byrt et al., 2014) implying that *larger quantities of Na^+ were staying in the root*. In this context, it will be interesting to compare if some key metabolic enzymes might have different affinity and/or sensitivity for Na^+ between root and shoot tissues.

Another possibility is that SOS1 Na^+/H^+ exchangers are not the key components of Na^+ extrusion from the root. GUS expression analysis in *Arabidopsis* revealed that SOS1 genes are expressed mainly in the root apex but not in the mature zone (Shi et al., 2002), so the net Na^+ efflux measured by the MIFE technique (Fig. 1) should account for all (most) of Na^+ extrusion, if the process is SOS1-mediated. However, the measured steady state Na^+ efflux in this work was only ~ 31% of unidirectional Na^+ influx (Suppl. Fig. S3), while the accepted view is that up to 95% of the total Na^+ taken by root is pumped back into rhizosphere (Munns, 2005). This leaves two possible options: either (contrary to GUS staining experiments) SOS1 exchangers are fully functional in the mature bulk of

the root, or Na^+ extrusion from root is mediated by some other transport mechanism that is not confined to root apex only. One of these transport mechanisms may be exocytosis. Exocytosis is the final event in the secretory pathway (Thiel and Battey, 1988). Genes of at least two members of exocyst families EXO70B1 and EXO70H7 are induced by salt stress in *Arabidopsis sos1-1* mutant roots (Oh et al., 2010), suggesting the possible involvement of an exocytosis pathway in the *sos1-1* roots under salt stress.



Suppl. Fig. 3 – Unidirectional Na^+ influx and Na^+ efflux in root elongation zone in barley Comparison of unidirectional Na^+ influx (calculated from Chen et al., 2007c) and Na^+ efflux (in the present study).

8.4.2 Transcriptional changes on gene expression do not account for functionality

In this study, although 2.5-folds higher Na^+ efflux in root elongation zone was found in salt tolerant (Gebeina) than sensitive (Unicorn) barley species under salt stress, no significant difference of relative expression level of *HvSOS1* was found between them (Fig. 8A) (Fig. 1C). One explanation can be assumed as the differential regulation of SOS1 by either SOS2/CIPK24 or SOS3/CBL4 between tolerant and sensitive varieties under salt stress. Another explanation may be due to the fact that SOS1 are involved in not only mediating Na^+ extrusion from the root to soil, but also loading Na^+ to xylem to be transferred to shoot (Olías et al., 2009b; Shabala, 2013; Maathuis et al., 2014). GUS expression analysis showed that SOS1 is expressed in the parenchyma cells surrounding the xylem (Shi et al., 2002). Constitutively higher shoot salt accumulation in the halophyte *Salicornia doluchostachya* than its taxonomically related glycophyte *Spinacia oleracea* was shown to be accomplished through enhancement of SOS1-mediated Na^+ xylem loading (Katschnig et al., 2015). Taken together, an increase of SOS1 in barley root elongation zone under salt stress can be partially interpreted as an increase of plant ability to load Na^+ to xylem. Combining functional analysis of real Na^+ extrusion ability with SOS1 gene expression analysis at cell specific level under salt stress is required in the future to investigate Na^+ extrusion further.

8.4.3 Potassium as a nutrient and a signalling agent

K^+ is the major cationic inorganic nutrient in non-halophytes, comprising generally 4 - 6% of plant dry matter (Pettigrew, 2008; Wakeel et al., 2011); it plays an important role in plant responses to major abiotic stresses such as drought, salinity, cold, frost, and waterlogging (Wang et al., 2013; Shabala and Pottosin 2014). As described earlier, K^+ retention ability in the root mature zone in many species was found to be significantly and positively correlated with the overall salt tolerance (Chen et al., 2005, 2007c). However, no such correlation was found in this study when measurements were conducted in the root elongation zone (Fig. 6D). Moreover, the overall NaCl-induced K^+ efflux from the elongation zone was nearly 2.5-fold higher than from the mature zone (-609 ± 38 nmol m^2s^{-1}, Fig. 6A; 45 varieties used vs -265 ± 10 nmol m^2s^{-1}, Chen et al., 2007d; 69 varieties used, same protocol as in the present study). What is the physiological rationale behind these observations?

One possible explanation may be that, given the difference in the size of the elongation and mature root zones, a 2.5-fold higher K^+ efflux from the former will have little impact on the overall root K^+ content but, at the same time, may play an important signaling role of a "metabolic switch" adapting plants to altered ionic conditions. Such idea has been recently proposed in the literature (Demidchik et al., 2014), and with over 70 enzymes being K^+ dependent (Anschutz et al., 2014) this hypothesis is highly plausible and worth further investigation. Amongst possible candidates one could mention proteins that are related to energy producing processes, such as succinate: CoA ligase (a critical enzyme in TCA cycle in mitochondria; Yokthongwattana et al., 2012) or pyruvate kinase (important for glycolysis; Veeranagamallaiah et al., 2008). It may be also suggested that the observed K^+ efflux should be relatively short lived (for not compromising the cell metabolism) and at some stage stopped and replenished from the vacuolar K^+ pool.

Interestingly, significantly higher relative expression level of *HvGork* in the root apex in salt sensitive barley varieties Unicorn (Fig. 8B) did not explain the lack of difference in NaCl-induced K^+ efflux in the root elongation zone between it and the salt-tolerant variety Gebeina (Fig. 6A). A possible explanation might be that GORK channel might not play the main role in mediating NaCl-induced K^+ efflux in the root elongation zone. Pretreatment with vanadate, a known inhibitor of H^+-ATPase, did not significantly change net steady state NaCl-induced K^+ efflux in the root elongation zone (Fig. 6B), suggesting that this process is not voltage gated. Keeping in mind a close association between salinity stress and ROS production (Bose et al., 2014b), the most likely candidate for this role may be some ROS-gated K^+ permeable channels such as the voltage-independent

non-selective cation channel (VI-NSCC; Demidchik and Maathuis, 2007). This is further supported by pharmacological observations in this and previous studies. Depolarization activated KOR channel dominated NaCl-induced K^+ efflux in the root mature zone (~80% inhibition, Fig. 7A and C). In the elongation zone, 60% of NaCl-induced K^+ flux was blocked by Gd^{3+} (Fig. 6B, Fig. 7B and D), a known blocker of NSCC.

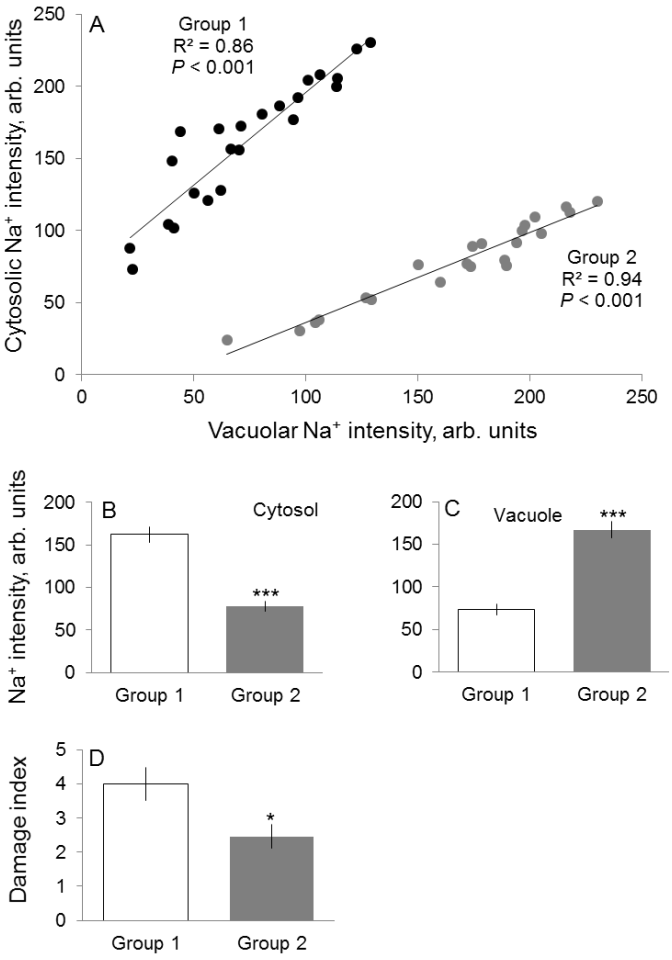
8.4.4 Increase in transcript levels of *HvNHX1* and *HvVP1* is not sufficient to achieve efficient vacuolar Na^+ sequestration

Vacuolar Na^+ sequestration is an important component of plant salt tolerance. In general, sequestrating Na^+ into the vacuole not only reduced cytosolic Na^+ level but also gave the opportunity to use the Na^+ as a cheap osmoticum. The classical view is that vacuolar Na^+ sequestration is achieved by NHX1 Na^+/H^+ exchanger which is expressed throughout seedlings as revealed by the GUS analysis (Bassil et al., 2011). Although a positive correlation between the cytosolic and vacuolar Na^+ intensity was found in the root elongation zone, increase of vacuolar Na^+ intensity in the relatively salt tolerant group is more insensitive to the increase of cytosolic Na^+ intensity than the relatively salt sensitive group (Suppl. Fig. S4).

At least three different types of transport systems contribute to vacuolar Na^+ sequestration (Shabala 2013). These include: (1) NHX1 Na^+/H^+ exchanger (Apse et al., 1999) that is responsible for pumping Na^+ into the vacuole; (2) vacuolar pumps (either H^+-APTases or H^+-PPase; Brini et al., 2007; Silva and Gerós, 2009) that energize the tonoplast and provide a driving force for NHX; and (3) slow (SV) and fast (FV) vacuolar channels that are permeable to Na^+ and should be kept close to prevent Na^+ back-leak into the vacuole (Bonales-Alatorre et al., 2013a, b). Under salt stress, the expression of *HvVP1* was shown to be coordinated with that of *HvNHX1* in barley at the whole root level (Fukuda et al., 2004a). However, in the root apex, we found that this coordinated pattern was shown in salt sensitive but not tolerant barley varieties (Fig. 8C and D), suggesting the tissue specific manner of coordination between regulation of *HvNHX1* and *HvVP1* under salt stress.

In the present study, vacuolar Na^+ sequestration ability (quantified by CoroNa Green fluorescence dye) was strongly ($r = 0.47$, $P < 0.01$) and positively correlated with the overall salt tolerance among barley accessions (Fig. 5E). However, contrary to our expectation, the salt sensitive barley variety showed several folds higher expression level of *HvNHX1* in root apex than the salt tolerant one (Fig. 8C). Also, significantly higher

relative expression level of *HvVP1* in the root apex was found in the salt sensitive than the salt tolerant variety (Fig. 8D). Thus, it appears that increased transcript levels of *HvNHX1* and *HvVP1* per se are not sufficient for achieving efficient vacuolar Na^+ sequestration. This can be either due to post-translational modifications (such as protein mis-folding etc), or due to an inability of sensitive varieties to control the permeability of FV or SV channels. Indeed, lower FV activity and SV density in mesophyll cell vacuole were shown in salt tolerant than sensitive quinoa (Bonales-Alatorre et al., 2013a), suggesting better ability to control Na^+ back-leak from the vacuole to cytosol in salt tolerant than sensitive plants. Also, FV currents were smaller in old quinoa leaves that accumulated more Na^+ than young ones (Bonales-Alatorre et al., 2013b).



Suppl. Fig. 4. Correlation analysis of Na^+ intensity between cytosol and vacuole in barley root elongation zone. (A) Correlation between cytosolic and vacuolar Na^+ intensity in barley root elongation zone. Cytosolic (B) and vacuoalr (C) Na^+ intensity, and damage index (D) between different groups.

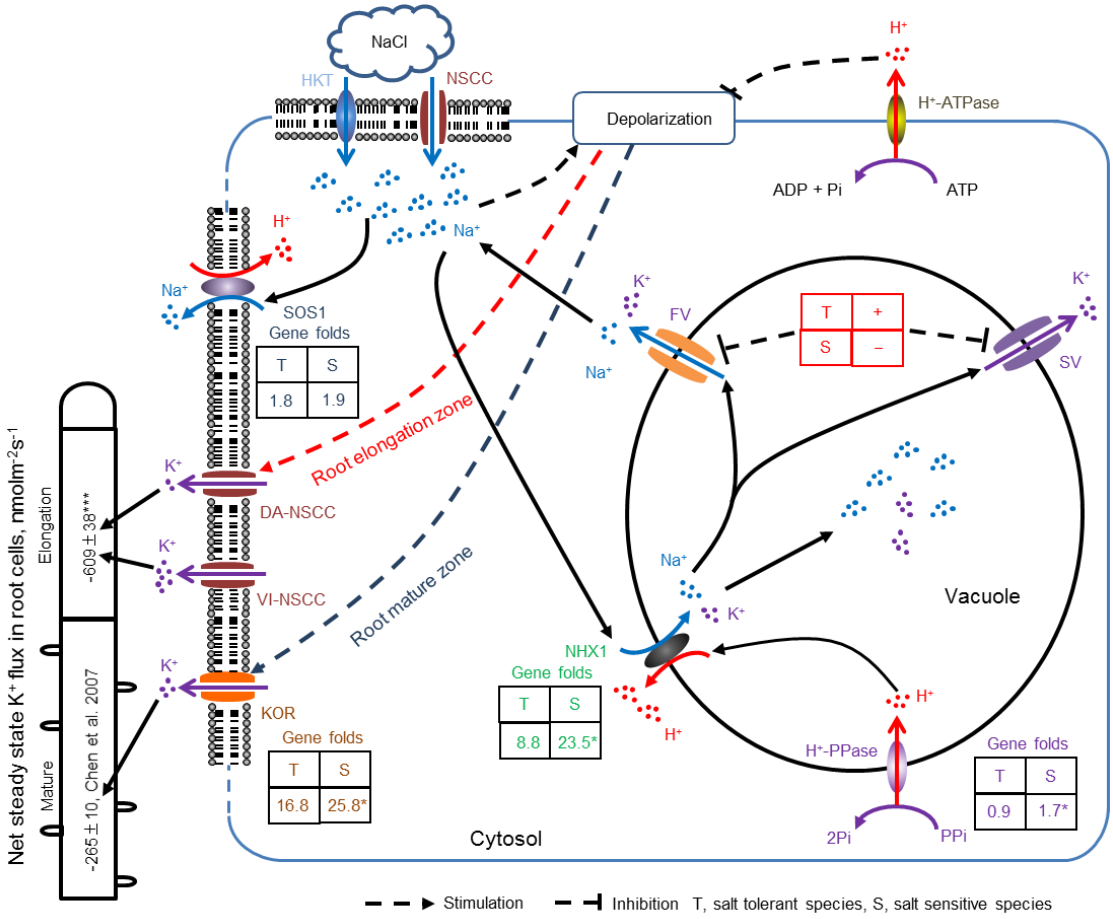


Fig. 9. Proposed model of cell specific salinity tolerance components in barley root elongation zone

8.4.5 The suggested model for the cation homeostasis in salinized roots

Fig. 9 summarizes key mechanisms involved in control of intracellular K^+/Na^+ homeostasis under salt stress in salinized barley roots. According to it, a massive Na^+ entry into cytosol via either NSCC channels or HKT transporters results in a rapid depolarization of membrane potential. Thus, even when Gd^{3+} was applied to block NSCCs (no 100% blockage can be reached), a certain amount of Na^+ can still can go into the cell cytosol causing depolarization of membrane potential and K^+ loss. GORK channels are activated in barley root mature zone to mediate NaCl-induced K^+ efflux. In the elongation zone, the main pathway for NaCl-induced K^+ efflux is through NSCC channel (either voltage-independent, VI-NSCC, or depolarization activated, DA-NSCC). The difference in this molecular identity of K^+ permeable channels between two zones is the main reasons for the 2.3-fold higher cytosol K^+ retention ability in the root mature zone than the elongation zone, since the root mature zone possesses better ability to restore its depolarized membrane potential under salt stress. Excessive Na^+ in the cytosol

can be either extruded from the cytosol to apoplast through SOS1 Na^+/H^+ antiporter (1.8- and 1.9-folds relative expression levels in salt tolerant and sensitive variety, respectively, $P > 0.05$) or sequestrated into vacuole by NHX1 Na^+/H^+ exchanger. Recently, NHX1 exchanger was shown to be mainly fuelled by the vacuolar H^+-PPase and the role of V-ATPase is limited for nutrient storage but not sodium accumulation (Krebs et al., 2010). However, although a much higher increase in the transcripts levels of both NHX1 exchanger (23.5- vs 8.8-folds, $P < 0.05$) and vacuolar H^+-PPase (1.7- vs 0.9-folds, $P <$ 005) were found in salt sensitive than tolerant varieties, sensitive varieties accumulated much less Na^+ in vacuoles, being unable to prevent Na^+ back-leak.

Chapter 9

Comparative analysis of Na^+ extrusion and sequestration patterns in roots of durum and bread wheat in the context of the long-distance salt stress signaling and adaptation [#]

Abstract

In this work, we investigated root Na^+ extrusion and sequestration patterns in wheat and linked it with its overall salt tolerance. Also, the possible role of the root meristem zone in salt stress sensing and long distance salt stress signal transduction was investigated. Bread wheat showed significantly higher vacuolar Na^+ sequestration ability than durum wheat but only in the root mature zone. No significant difference of vacuolar Na^+ sequestration was found in either root meristem or transition zone. Interestingly, durum wheat showed significant higher vacuolar Na^+ sequestration than bread wheat in the root elongation zone, suggesting a compensation mechanism to offset the lower ability to extrude Na^+ from the cytosol to apoplast than bread wheat. Although no significant difference of leaf Na^+ content was found, intact plants (with intact roots) showed significantly higher chlorophyll content and F_v/F_m than the cut plants (with the removal of roots meristem zone). Significantly higher relative expression level of both *TaNHX1* and *TaVP* was found in intact plants than the cut plants under salt stress. It indicates that leaf Na^+ distribution under salt stress was affected by the removal of root meristem zone, suggesting that the root meristem zone played a role in salt stress sensing and long distance salt stress signal transduction from root to shoot.

Keywords

Na^+ extrusion, root meristem zone removal, root zones, salt stress sensing, vacuolar Na^+ sequestration

[#], This chapter will be submitted as: Wu H, Shabala L, Zhou M, Mancuso S, Azzarello E, Shabala S: New Phytologist.

9.1 Introduction

Bread wheat is always considered as more salt tolerant than durum wheat (Munns and James, 2003; Munns and Tester, 2008), and this difference is mainly attributed to its superior ability to maintain lower Na^+ accumulation in leaf/shoot (Colmer et al., 2006; Cuin et al., 2010; James et al., 2011; Munns et al., 2012). However, it remains unclear whether this pattern is achieved by stronger shoot Na^+ exclusion traits such as better control of Na^+ loading into the xylem (Davenport et al., 2005; Lauchli et al., 2008, Zhu et al., 2015) or increased Na^+ retrieval from the shoot (James et al., 2006a; Davenport et al., 2007), or is due to reduced net root Na^+ uptake *per se*. Moreover, some recent studies showed the lack of significant correlation between shoot Na^+ exclusion ability and salt tolerance in Arabidopsis (Rus et al. 2006, Jha et al., 2010), bread wheat (Genc et al. 2007), and tomato (Almeida et al. 2014), pointing out the importance of Na^+ sequestration in the shoot (so-called tissue tolerance; Munns and Tester 2008) as arguably the most essential component of salinity stress tolerance. Moreover, as commented in Chapter 8, most authors have a tendency to use the term "sodium exclusion" in a very broad term, and often in the context of prevention of its accumulation in the shoot/leaf. As a result, the actual role of Na^+ extrusion from the root, and its contribution to the differential salt tolerance between bread and durum wheat remains largely unknown. At the very best, a comparison was done by monitoring $^{22}Na^+$ uptake in radiotracer experiments (Davenport et al., 1997). These studies have revealed that no significant difference of $^{22}Na^+$ content in both shoot and root was found between salt tolerant bread wheat Kharchia and salt sensitive durum wheat Modoc. To the best of our knowledge, the comparative analysis of the activity of SOS1-like Na^+ efflux systems between durum and bread wheat has never been conducted, at the large scale functional level. Meanwhile, insights into this issue might be beneficial for improving plant salt tolerance since in most case the accumulated excessive Na^+ in cytosol cause the Na^+ toxicity.

There is a growing bulk of evidence suggesting that tissue specificity of stress sensing and adaptation to salinity may be a key to understanding the complexity of the overall plant salt tolerance (Verdoy et al., 2004; Wu et al., 2015a). Tissue specific expression patterns of genes in plants under salt stress have been widely reported (Claes et al., 1990; Low et al., 1996; Saijo et al., 2000; Jain et al., 2007; Dinneny et al., 2008; Iyer-Pascuzzi et al., 2011). Also, in *Arabidopsis* root, Ca^{2+} responses to salt stress were shown to be highly cell type specific (Kiegle et al., 2000). As cytosolic Na^+ toxicity is one of the main

reasons for adverse salinity effects on plant performance (Tester and Davenport, 2003; Munns and Tester, 2008). A knowledge of Na^+ distribution in cell compartments (i.e. cytosol and vacuole) can be regarded as a potential indicator for plant salt tolerance. However, so far, not much attention has been paid to the possible difference in Na^+ distribution in various cell compartments at the cell type specific level in wheat roots.

The importance of vacuolar Na^+ sequestration for overall salt tolerance is widely accepted (Apse et al., 1999, Zhang et al., 2001). Improved salt tolerance from overexpressing the NHX1 K^+, Na^+/H^+ exchanger has been reported in many species such as Arabidopsis (Apse et al., 1999), wheat (Xue et al., 2004), rice (Chen et al., 2007), cotton (He et al., 2007), tomato (Zhang and Blumwald, 2001), maize (Yin et al., 2004), peanut (Banjara et al., 2012), tobacco (Gouiaa et al., 2012), and *Brassica napus* (Zhang et al., 2001). However, most of the studies on the role of vacuolar Na^+ sequestration in plant salt tolerance are conducted at the whole plant or leaf/shoot level with only a few papers (e.g. Zhang et al., 2001; Chen et al., 2007a) on roots. In the latter case, all of the reported root Na^+ concentration in the transgenic line is on overall bulk root data, no attention was paid to tissue-specific aspects. Less attention was paid to the tissue specific role of vacuolar Na^+ sequestration in the overall plant salt tolerance. Using six contrasting bread wheat varieties we have recently shown (Wu et al. 2015) that the salt tolerant group showed significant higher vacuolar Na^+ sequestration ability in the mature root zone but not the root apex than the salt sensitive group, under saline conditions. Whether the importance of the vacuolar Na^+ sequestration in root mature zone can be extrapolated from one subspecies (bread wheat) to different subspecies (bread and durum wheat) needs to be clarified.

So far, the knowledge of how plants perceive the salt stress, transfer the stress signal to whole plant level, and coordinate all adaptive responses at the whole-plant level to deal with the hostile soil conditions remains largely obscure. In animals, Na^+ sensing mechanism appears to consist mostly of specific Na^+ selective ion channels e.g. Na_X channels and other Na^+ transporters e.g. Na^+ dependent glutamate transporters (Shimizu et al., 2007; Maathuis, 2014). In our seminal paper (Wu et al. 2015), we found that the salt tolerant group possesses higher cytosolic Na^+ intensity in the root meristem zone compared with the salt sensitive group. This led to the suggestion that the root meristem in bread wheat might participate in, or execute, a role as the salt sensor. To validate our hypothesis about the role the root meristem zone plays in salt sensing, more direct evidence is needed.

In this work, we used a broad range of bread and durum wheat accessions to quantify Na^+ distribution between the cytosol and vacuole in four different root zones - root meristem; transition zone; elongation zone; and mature root zone – using fluorescent imaging microscopy. Also, using MIFE (non-invasive microelectrode ion flux estimation system) technique we have quantified, for the first time, the contribution of Na^+ extrusion in the root elongation zone in the differential salinity stress tolerance in bread and durum wheat. Finally, we have investigated the effects of the removal of root meristem (containing the tentative Na^+ sensor) on plant phenotype salt stress signalling and adaptation. Our results show: 1) the tissue specificity of vacuolar Na^+ sequestration in different root zones in overall salt tolerance in bread and durum wheat, 2) Na^+ extrusion in the root elongation zone is correlated with overall salt tolerance in bread and durum wheat, 3) the potential role of salt sensor by the root meristem zone can be expanded from one subspecies (bread wheat) to two subspecies (bread and durum wheat), 4) a long distance salt stress signal transduction in salinized plants after the removal of the root meristem zone.

9.2 Materials and methods

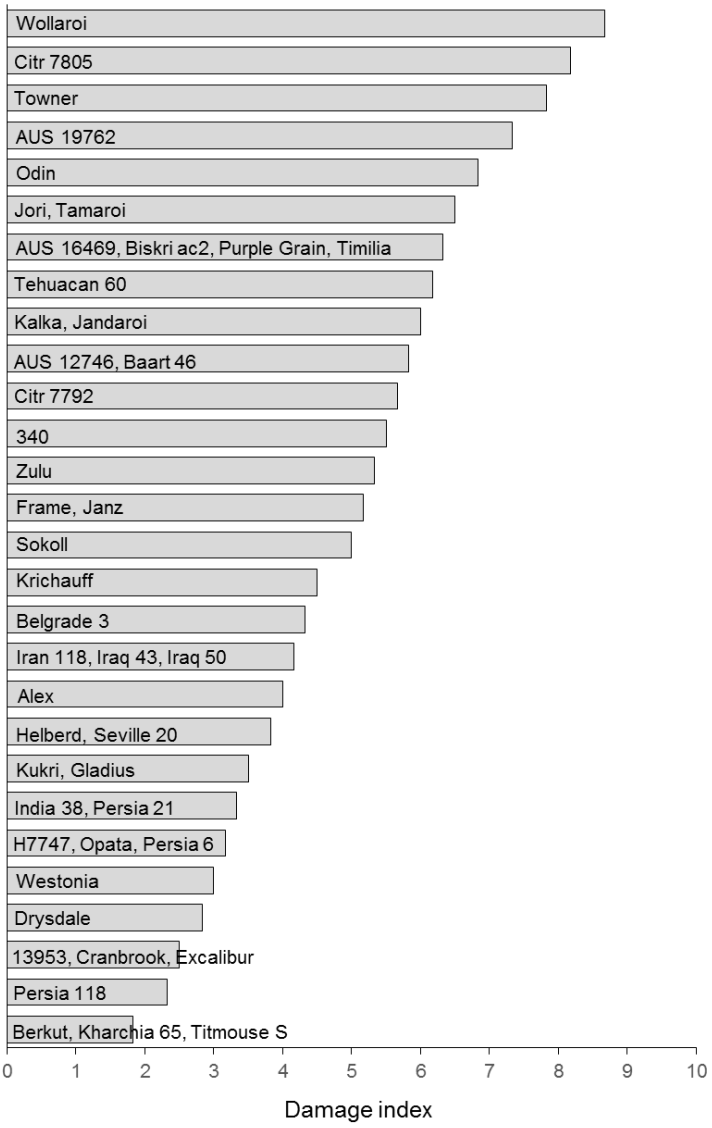
9.2.1 Plant growth and conditions

27 bread wheat (*Triticum aestivum*) and 19 durum wheat (*Triticum turgidum* spp. durum) were used in this study. All seeds were obtained from different sources and multiplied in our laboratory. Twenty seeds for each variety were surface sterilized with 5% commercial bleach for 10 min, and then washed under running tap water for about 0.5 h. Twelve to 16 uniform seeds were grown in a l L PVC container with double distilled water (ddH_2O). Good aeration was provided with aquarium stones connected to an air pump (Aqua One® 9500). Seedlings were grown in darkness at 25 ± 2 °C for 3 days followed by the salinity treatment (100 mM NaCl) for 24 h. Salt stressed seedlings were used to investigate the Na^+ extrusion ability of wheat roots.

9.2.2 Glasshouse experiments

Effect of salinity on plant performance was investigated in glasshouse experiments. Plants were grown in the glasshouse facilities at the University of Tasmania. Twelve to 14 seeds of each variety were sown in a 4.5 L PVC pot with standard potting mix (Potting mix: 80% composted pine bark, 10% sand and 10% coir peat, plus complete N:P:K (8:4:10), 1 Kg m^{-3}; dolomite, 8 Kg m^{-3}; gypsum, 1 Kg m^{-3}; iron sulphate, 1 Kg m^{-3};

isobutylenediurea, 1 Kg m^{-3}; trace element mix, 0.75 Kg m^{-3}; wetting agent, 0.75 Kg m^{-3}; zeolite, 0.75 Kg m^{-3}; pH 6.0.) Each experiment was repeated in triplicate. After 6 days, the plant numbers in each pot were uniformly thinned to eight plants, and salt treatment (300 mM NaCl) was applied for further 38 days. A saucer was placed under each pot and plants were irrigated twice a day by an automatic watering system with dripper outlets for both control condition and salinity treatment. The damage index (quantified on 0 to 10 scale and based on the extent of leaf chlorosis and plant survival rate; Zhou et al. 2012, Wu et al., 2014, 2015) was scored before harvesting (Suppl. Fig. S1). The higher damage index score represents the lower salinity tolerance.



Suppl. Fig. 1 – Genetic variability of damage index under salt stress
Damage index of wheat varieties grown in 300 mM NaCl for 38 days.

9.2.3 Preparation of ion selective microelectrodes for non-invasive ion flux measurement

Net Na^+ and H^+ fluxes from excised roots after removal of external salt were measured using non-invasive microelectrode ion flux estimation technique (the MIFE; Shabala et al. 2006). For MIFE preparation, blank microelectrodes were pulled out from borosilicate glass capillaries (GC 150-10; Harvard Apparatus, Kent, UK), dried in an oven (at 225 ºC, overnight), and silanized with tributylchlorosilane (No 282707, Sigma-Aldrich, St. Luis, MO, USA). The silanized microelectrode blanks were filled from the back with an appropriate backfilling solution (for H^+: 15 mM NaCl + 40 mM KH2PO4; pH 6.0 adjusted using NaOH and for Na^+: 0.5 M NaCl) followed by front filling the tip with a respective Liquid Ion Exchanger (LIX). A commercially available LIX was used for H^+ (catalogue No 95297, Sigma-Aldrich, St. Luis, MO, USA) while LIX for Na^+ was prepared in the laboratory as described earlier in the publication (Jayakannan et al. 2011). The prepared microelectrodes were mounted in MIFE electrode holders and calibrated in a set of three respective standards before and after use. Only microelectrodes with a slope above 50 mV per decade and correlation over 0.999 were used.

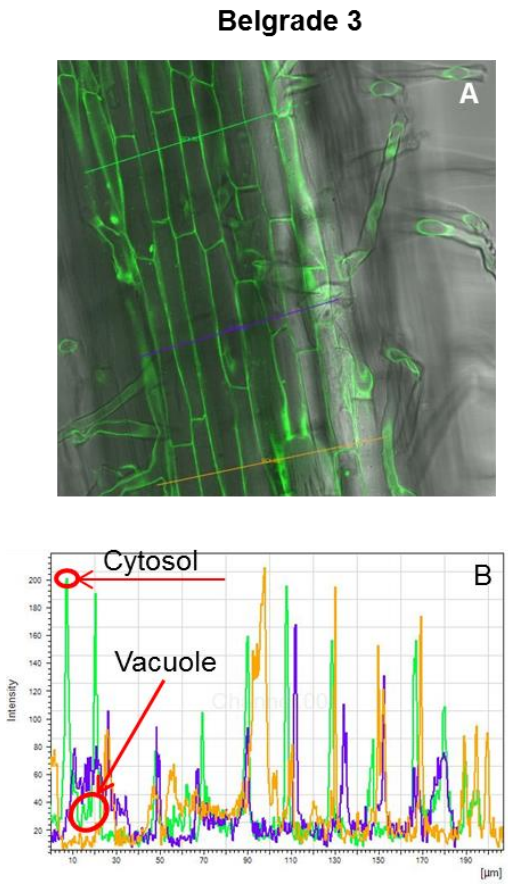
9.2.4 Screening of Na^+ extrusion ability in wheat root via MIFE technique

A rapid method was established to screen root Na^+ extrusion in a large number of wheat genotypes (46 varieties) by using MIFE technique to measure Na^+ efflux from the root elongation zone after removal of external salt. The salt treated plants (see the "Plant growth and conditions") were gently rinsed in a beaker containing 10 mM $CaCl_2$ for 1 min followed by gentle rinsing in ddH_2O for another minute. About 3cm long root segments were cut starting from the root tip. Excised root segments were then mounted in a fixed perspex holder in a petri dish containing ddH_2O (10 mL) for adaptation for 0.5 h. Using an inverted microscope, Na^+ and H^+ selective microelectrode tips were positioned a few micrometers apart and in one plane with the root segment to be assessed. Initial distance between the microelectrode tips and an excised root segment was adjusted to 40 μm and the microelectrodes were moved 110 μm away and back to the starting position during the measurements by a computer-controlled hydraulic manipulator staying for 6 secs in each position (i.e. in a 12 secs square-wave cycle). In each experiment an electrochemical gradient potential was recorded for 5 min and converted into net ion

fluxes using the calibration values of the ion selective microelectrodes and cylindrical diffusion geometry of the root (MIFEFLUX software, Newman 2001, Shabala et al. 2006). At least six root segments were assessed for each treatment and variety.

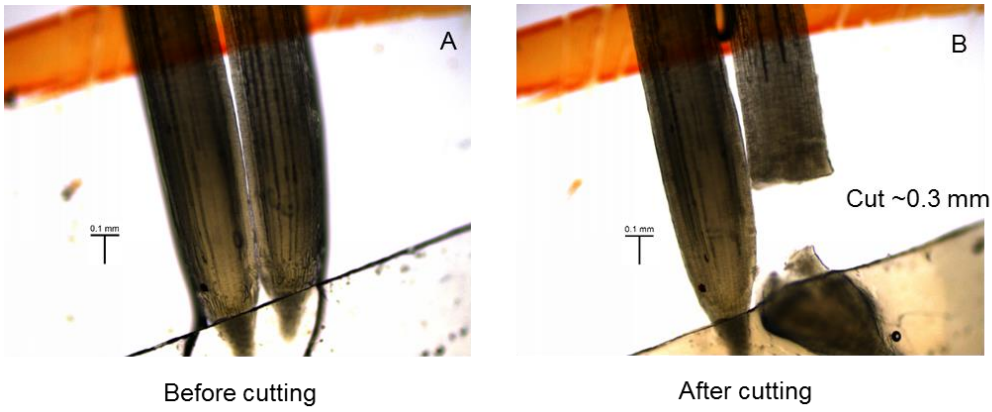
9.2.5 Screening Na^+ distribution in vacuolar and cytosolic compartments in different root zones in wheat by LSCM

Green fluorescent Na^+ dye CoroNa Green acetoxymethyl ester (Cat.C36676, Invitrogen) was used to assess the amount of Na^+ accumulated in cytosol and vacuoles of the different wheat root zones, essentially as described in our previous publications (Bonales-Alatorre et al. 2013b, Wu et al. 2015). Briefly, roots were cut into two segments, mature (30 to 40 mm from the apex) and elongation (the first 10 mm from the apex) zones, incubated in a solution containing 20 μM CoroNa Green for 2h in the dark, rinsed in a buffered MES solution (5 mM MES, pH 6.3 adjusted with KOH), and examined using an upright Leica Laser Scanning Confocal Microscope SP5 (Leica Microsystems, Germany) equipped with a 40× oil immersion objective (excitation wavelength, 488 nm; emission, 510-520 nm). LAS Lite software (Leica Microsystems, Germany) was used for image analyses (Suppl. Fig. S2). Comparison of different fluorescence intensity was carried out by visualizing cells with the identical imaging settings on the confocal microscope. The mean fluorescence intensity values for cytosolic and vacuolar compartments were then calculated for each cell. Light microscopy images (visualising root cell morphology) were used to clarify the signal profile distribution in the cytosol and vacuole. Readings from between 60 to 100 cells were averaged and reported for each genotype. Root meristem and mature zones were distinguished by root morphology. The identification of root transition and elongation zones was carried out by using another fluorescent dye FM4-64 (Cat: T-3166, ThermoFisher Scientific) to visualize tonoplast membrane (see details in Wu et al. 2015).



Suppl. Fig. 2 Illustration of the quantification method for Na^+ distribution in the cytosol and vacuole. (A) Several lines are drawn across the so-called "region of interest" (ROI) in an appropriate root zone. (B) Continuous fluorescence intensity distribution profiles obtained by LAS AF Lite software. The mean fluorescence intensity values for the cytosol and vacuole are then calculated for each cell by attributing signal profiles to root morphology (visualised by light microscopy images). The mature root zone of bread wheat Belgrade 3 was used.

9.2.6 Plant performance under salt stress after removing root meristem zone



Suppl. Fig. 3. Removal of the root meristem zone. The root meristem zone was removed under a microscope. (A) Before removal of the root meristem zone, (B) After removal of the root meristem zone.

Twelve to 16 uniform seeds of salt tolerant bread wheat (variety Kharchia 65) were grown in a l L PVC container with a perforated plastic insert for seeds. The container was filled with ddH_2O and seedlings were grown with aeration for 3-4 days in the dark. Seedling numbers were thinned to 6 uniform plants in each pot. At the commencement of

measurements all of the root meristem zones were carefully removed (~ 0.3 mm from the root tip) from each plant under a microscope (Suppl. Fig. S3). Plants were then transferred back into containers with ¼ Hoagland solution and grown for a further 10 days in the presence and absence of 200 mM NaCl. A number of physiological parameters were measured at the end of the experiment including: leaf chlorophyll content index CCI (measured by a chlorophyll meter SPAD-502, Konica Minolta, Tokyo, Japan), the maximal photochemical efficiency of photosystem II F_v/F_m (quantified by a chlorophyll fluorometer OS-30p, Optisciences, Hudson, NH, USA), and shoot and root FW (plants were rinsed in ddH_2O and the possible residues of salt on leaves were gently washed out). Samples were dried at 65 °C for 72 h in a Unitherm Dryer (Birmingham, UK) and dry weight (DW) of shoots and roots was estimated. During salt stress treatment, lateral roots in salt stressed plants were checked and eliminated every day. During the salt treatment the PVC containers were kept under ~150 μmol m^{-2} s^{-1} irradiance at 26/20 ± 1.0 °C day/night temperatures, 16 h/8 h day/night cycle.

Root cutting experiments were also conducted using potting mix in a glass house (October 2015). For this, seedlings were grown in paper rolls in containers with ddH_2O for four days. The root meristem zone was removed under a microscope as described above. Then the intact plants (with intact roots) and cut plants (with removed root meristems) were transplanted to a 1.7 L pot filled with standard potting mix. A saucer was placed under each pot. Hand watering of all the pots with tap water for 2 days was used to allow the adaption of the transplanted plants. Then, the plants were uniformly thinned to 5-7 plants in each pot and salt stress (200 mM NaCl) was applied for 15 days. Possible effect of kinetin (Shabala et al., 2009) was tested by growing plants in the presence or absence of 4 μM kinetin for 15 days under saline (200 mM NaCl) and control (tap water) conditions. At the end of the experiment, CCI of each plant and shoot FW were measured. For FW estimation, plants were cut 1 cm above the potting mix and the FW of collective samples from each pot was immediately measured.

9.2.7 Estimation of leaf and root Na^+ content

Dry leaf and root samples were digested in a 120-mL Teflon digestion vessel in a microwave digester (Mars 6 one touch microwave digestion system, CEM Corporation, Matthews, NC, USA) using 7 mL of 70 % HNO3 per 0.1 g specimen. Digested samples were centrifuged at 5000g for 10 min at room temperature (Avanti J-30I centrifuge, Beckman Coulter, Krefeld, Germany). After centrifugation, 0.2 mL of the digested

solution was diluted with ddH_2O to a final volume of 10 mL. Na^+ content was then measured using a Flame Photometer (PFP7, Jenway, UK).

9.2.8 Quantitative Real-time PCR Analysis

Target genes	Locus	Primer sequences
TaNHX1	AY040245	F: 5'-GCCTGGTTCACCCATAGAGA-3'
		R: 5'-CACCGAAAGAATCCCAAGAG-3'
TaVP1	EU255237.1	F: 5'-CCTATCTTCGCCATTGCCTTC-3'
		R: 5'-CAGCATCCAGAGCATCAGTTC-3'
TaSOS1	AY326952	F: 5'-ATTCCCTCAGGTGCTTCGTG-3'
		R: 5'-TTTCCTCGAGCAACCCAGTC -3'
TaHA1	AY543630.1	F: 5'-GCTGATTGAGAAGGCTGATGG-3'
		R: 5'-TCGGTAAGCACAATGTCTGAAG-3'
TaActin	AF326781	F: 5'- TACACGAAGCGACATACAA-3'
		R: 5'-AATAGAGCCACCGATCCA-3'

Suppl. Table 1. Primers used in this study.

Plants with intact and cut roots grown in ¼ Hoagland solution in the absence and presence of 200 mM NaCl were used. The youngest fully expanded leaves of 2 weeks old wheat seedlings (Kharchia 65) treated with 200 mM NaCl for 10 days were harvested and snap frozen in liquid nitrogen. The leaf RNA was extracted and synthesised to cDNA by using RNeasy Mini Kit (QIAGEN) and QuantiTect® Reverse Transciption Kit (QIAGEN) following the manufacture's instruction. Quantitative real-time PCR was performed using a RG6000 Rotor-Gene Q Real Time Thermal Cycler and SYBR green PCR reagent (QIAGEN) as described in Vandesompele et al. (2002). A $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) was used to analyse the relative expression levels of studied genes. Primers were designed to determine the expression of *TaSOS1*, *TaNHX1*, *TaVP*, and *TaHA1* (please refer to Suppl. Table S1 for primer sequences). The control gene (*TaActin*) was used for normalization of the test gene transcript. Experiments were repeated three times with consistent results.

9.2.9 Data analysis

All data (given as mean ± SE, n = sample size) were analysed by using SPSS 20.0 for windows (SPSS Inc., Chicago, IL, USA). All of the replicates were biological replicates. Comparison of different parameters between bread and durum wheat were performed by independent samples t-test and oneway ANOVA based on Duncan's multiple range test. The symbol "*" states for $P < 0.05$ and, "**" states for $P < 0.01$. The "NS" means $P >$

0.05. Different lower case letters represent the significance at $P < 0.05$, whereas the same lower case letters indicate no significant difference. The significance of correlations between different parameters was determined by Bivariate Correlations based on Pearson Correlation (2-tailed). The data used for correlation analysis are the average values of measured independent parameters for each variety.

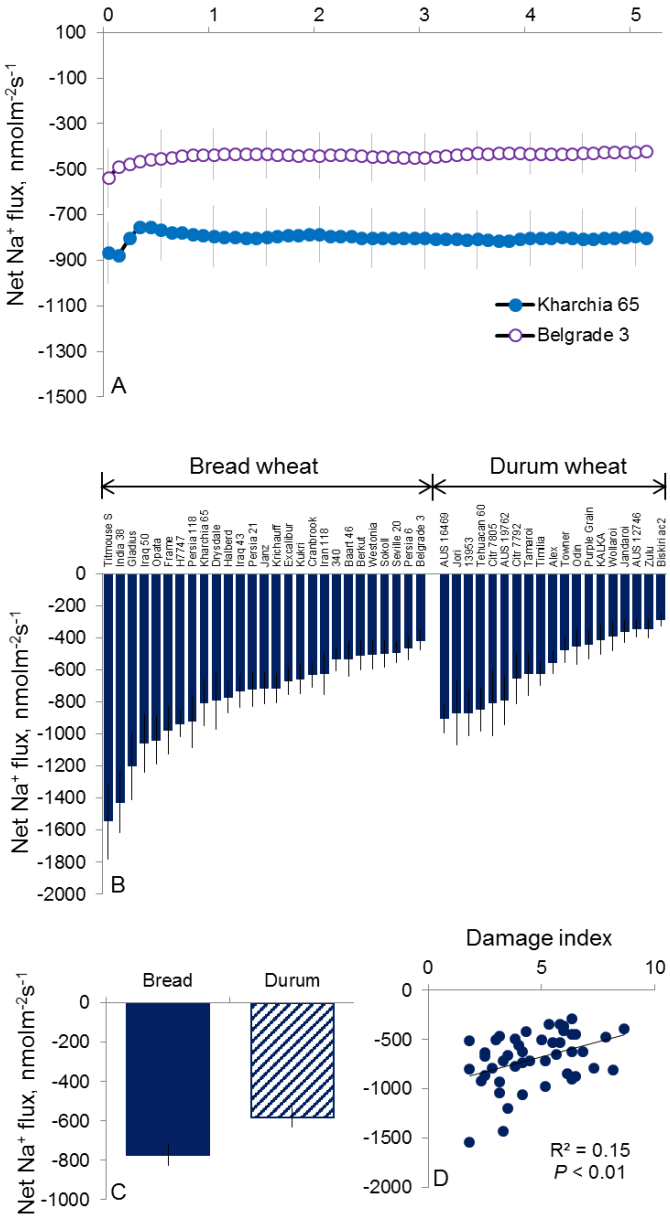


Fig. 1. Genetic variability of Na^+ efflux in the root elongation zone after the removal of external salt. (A) The kinetics of net steady state Na^+ efflux in the root elongation zone after the removal of external salt in bread wheat (cv Kharchia 65 and Belgrade 3). Mean ± SE (n= 6 − 13). (B) Net steady state Na^+ efflux in 46 wheat varieties. Mean ± SE (n= 6 − 13). (C) Pooled mean values of average Na^+ efflux for bread and durum wheat clusters. Mean ± SE (n= 19 (durum wheat) and 27 (bread wheat)).

9.3 Results

9.3.1 Significant higher Na^+ extrusion in the root elongation zone in bread than durum wheat

Representative steady state Na^+ efflux from the root elongation zone after removal of external salt is shown in Fig. 1A. Over 5.4-fold variability was found among the screened 46 wheat varieties, with the highest Na^+ efflux being -1584.4 ± 237.2 (in Titmouse S, bread wheat) and the lowest Na^+ efflux of -284.0 ± 39.6 nmol m^{-2} s^{-1} (in Biskiri ac2, durum wheat) (Fig. 1B). On average, 33% higher ($P < 0.05$) Na^+ extrusion ability from the root elongation zone was found in bread (-773.2 ± 55.4 nmol m^{-2} s^{-1}) than durum wheat (-580.5 ± 48.6 nmol m^{-2} s^{-1}) (Fig 1C). A strong and positive correlation was observed between Na^+ extrusion and plant overall salinity stress tolerance ($r = 0.39$, $P < 0.01$; Fig. 1D).

9.3.2 Cytosolic Na^+ accumulation in root meristem zone was significantly higher in bread wheat

Representative images of Na^+ distribution between cytosol and vacuole in different root zones in bread (Gladius) and durum (Purple Grain) wheat is shown in Fig. 2-5. A 16.7-fold variability in cytosolic Na^+ intensity was observed within the screened 46 wheat varieties, ranging from the highest 174.0 ± 6.7 (Cranbrook, bread wheat) to lowest 10.4 ± 0.4 (Persia 21, bread wheat) (Fig. 2E). Similarly, a large variability (5.4-fold) of vacuolar Na^+ intensity in the root meristem zone was found within the screened wheat, ranging from the highest value of 179.9 ± 6.6 (Wollaroi, durum wheat) to the lowest of 33.5 ± 2.0 (Iraq 50, bread wheat) (Fig. 2G). Still, significantly higher cytosolic Na^+ intensity in the root meristem zone was found in bread rather than durum wheat (57.9 ± 9.8 vs 31.7 ± 2.3, $P < 0.05$; Fig 2F). At the same time no significant difference was found in vacuolar Na^+ intensities between bread and durum wheat (84.8 ± 6.4 vs 102.6 ± 6.8, respectively, $P > 0.05$; Fig. 2H). Also, a significant positive correlation ($r = 0.36$, $P < 0.05$; Fig. 6A) was found between cytosolic Na^+ intensity in the root meristem zone and overall salt tolerance in wheat, compared with no correlation ($r = 0.14$, $P > 0.05$; Fig. 6B) in vacuolar Na^+ intensity in the root meristem zone.

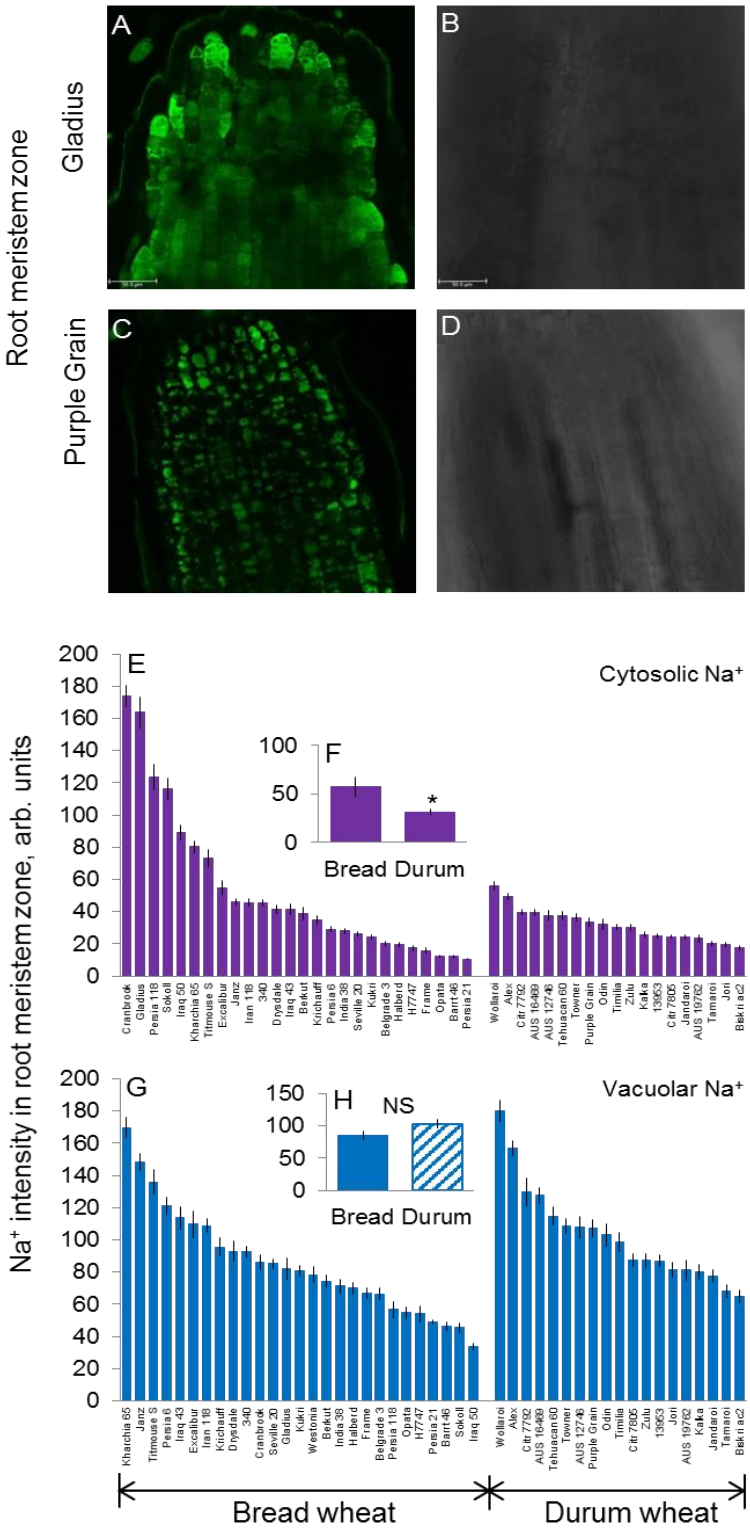


Fig. 2. Genetic variability of Na^+ intensity in the cytosol and vacuole in the root meristem zone in wheat. Representative CoroNa Green dye images in the root meristem zone in bread wheat Gladius (A) and durum wheat Purple Grain (C). B and D show the corresponding light images. Intensity of CoroNa Green fluorescence (arb. units) in the cytosol (E) and vacuole (G) in the root meristem zone of 46 wheat varieties contrasting in their salinity stress tolerance. Mean ± SE (n= 72 – 96 cells from at least 6 individual plants). Averaged pooled values for Na^+ intensity in the cytosol (F) and vacuole (H) in the root meristem zone for bread and durum wheat clusters. Mean ± SE (n= 19 (durum wheat) and 27 (bread wheat)).

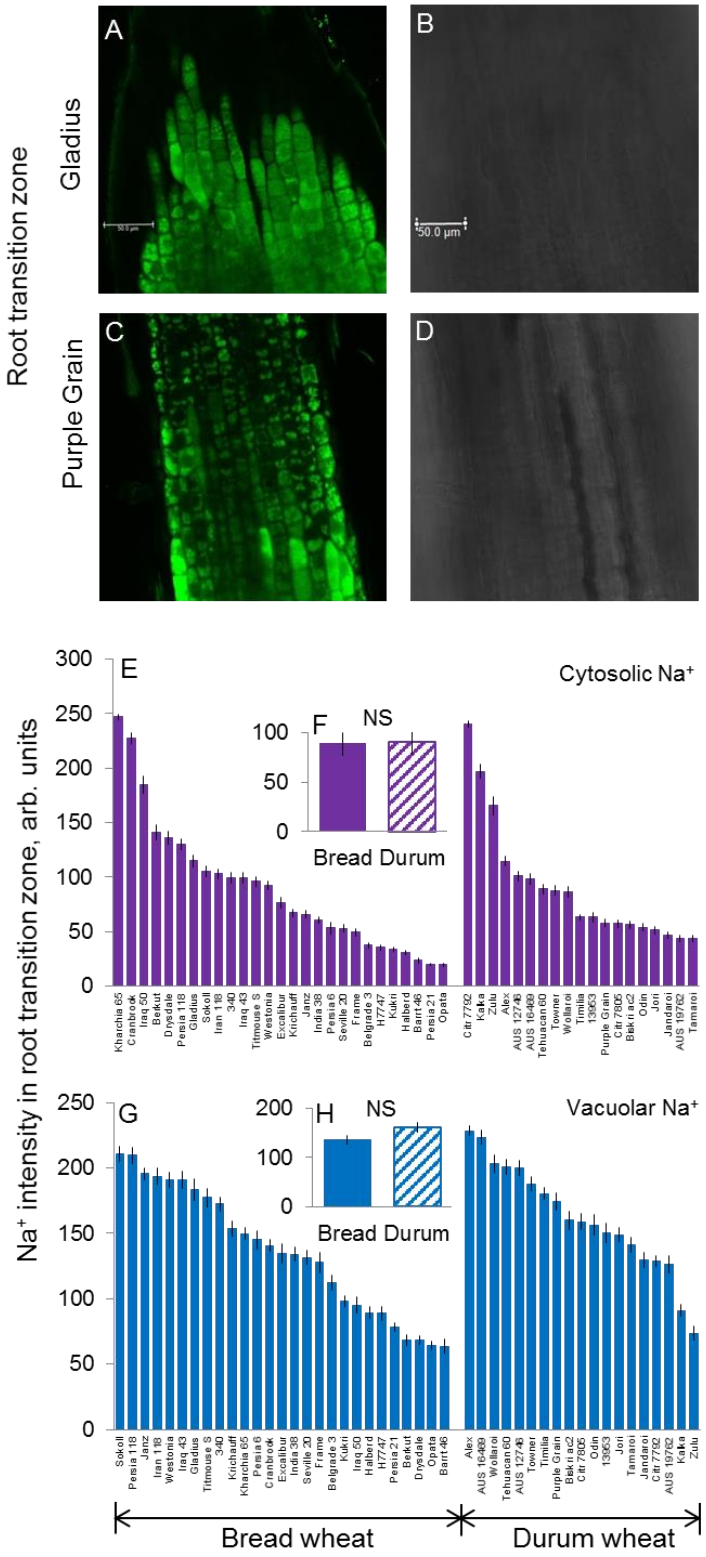


Fig. 3. Genetic variability of Na^+ intensity in the cytosol and vacuole in the root transition zone in wheat. Representative CoroNa Green dye images in the root transition zone in bread wheat Gladius (A) and durum wheat Purple Grain (C). B and D show the corresponding light images. Intensity of CoroNa Green fluorescence (arb. units) in the cytosol (E) and vacuole (G) in the root transition zone of 46 wheat varieties contrasting in their salinity stress tolerance. Mean ± SE (n= 72 – 96 cells from at least 6 individual plants). Averaged pooled values for Na^+ intensity in the cytosol (F) and vacuole (H) in the root transition zone for bread and durum wheat clusters. Mean ± SE (n= 19 (durum wheat) and 27 (bread wheat)).

9.3.3 Na^+ distribution in the root transition zone was not different between bread and durum wheat

Na^+ distribution in the cytosol and vacuole of the root transition zone is shown for a bread wheat (Gladius, Fig. 3A, B) and durum wheat (Purple Grain, Fig. 3C, D). Big variability of Na^+ intensities in the cytosol and vacuole of the root transition zone was found in bread (12.6-fold) and durum wheat (3.5-fold). Bread wheat Kharchia 65 showed the highest cytosolic Na^+ intensity in the root transition zone (247.3 ± 2.5), compared with the lowest value of 19.7 ± 1.3 found also in bread wheat Opata (Fig. 3E). Similarly, in the vacuole the highest Na^+ intensity of 228.4 ± 3.9 was found in the root transition zone of a durum wheat variety Alex compared to the lowest value of 63.7 ± 5.2 found in the bread wheat Baart 46 (Fig. 3G). When averaged, no significant difference of Na^+ intensity in either the cytosol (bread wheat 89.1 ± 11.5 vs durum wheat 90.6 ± 12.6, $P > 0.05$; Fig. 3F) nor vacuole (bread wheat 135.8 ± 9.3 vs durum wheat 161.2 ± 9.5, $P > 0.05$; Fig. 3H) was found between bread and durum wheat root transition zone. Also, no significant correlation was found between overall salt tolerance and either cytosolic ($r = 0.22$, $P > 0.05$; Fig. 6C) nor vacuolar Na^+ intensity ($r = 0.27$, $P > 0.05$; Fig. 6D) in the root transition zone.

9.3.4 Durum wheat possesses superior vacuolar Na^+ sequestration ability in root elongation zone

The profile of Na^+ distribution between the cytosol and vacuole in the root elongation zone is shown in Fig. 4A-D. A large variability (5.8-fold) of cytosolic Na^+ intensity was observed in all 46 wheat varieties tested, ranging from the highest value of 250.3 ± 1.9 (Kalka, durum wheat) to the lowest one of 43.2 ± 2.2 (Kukri, bread wheat) (Fig. 4E). No significant difference of cytosolic Na^+ intensity in the root elongation zone was found between bread and durum wheat (160.5 ± 14.0 and 169.0 ± 11.7, respectively; $P > 0.05$; Fig. 4F). Similarly, 3-fold variability of vacuolar Na^+ intensity was found in the root elongation zone within the screened 46 wheat varieties (Fig. 4G). It ranged from the highest value of 254.3 ± 0.3 (Jandaroi, durum wheat) to the lowest one of 83.5 ± 5.0 (Halberd, bread wheat) (Fig. 4G). Interestingly, significantly ($P < 0.01$) lower vacuolar Na^+ intensity in the root elongation zone was found in bread (157.4 ± 8.8) rather than durum wheat (205.4 ± 12.1) (Fig. 4H). In tune with this, a strong and negative correlation ($r = 0.41$, $P < 0.01$) was found between vacuolar Na^+ intensity in the root elongation zone and the overall salinity tolerance in wheat (Fig. 6F), compared with no or weak

correlation (r = 0.14, $P > 0.05$) in cytosolic Na^+ intensity in root elongation zone (Fig. 6E).

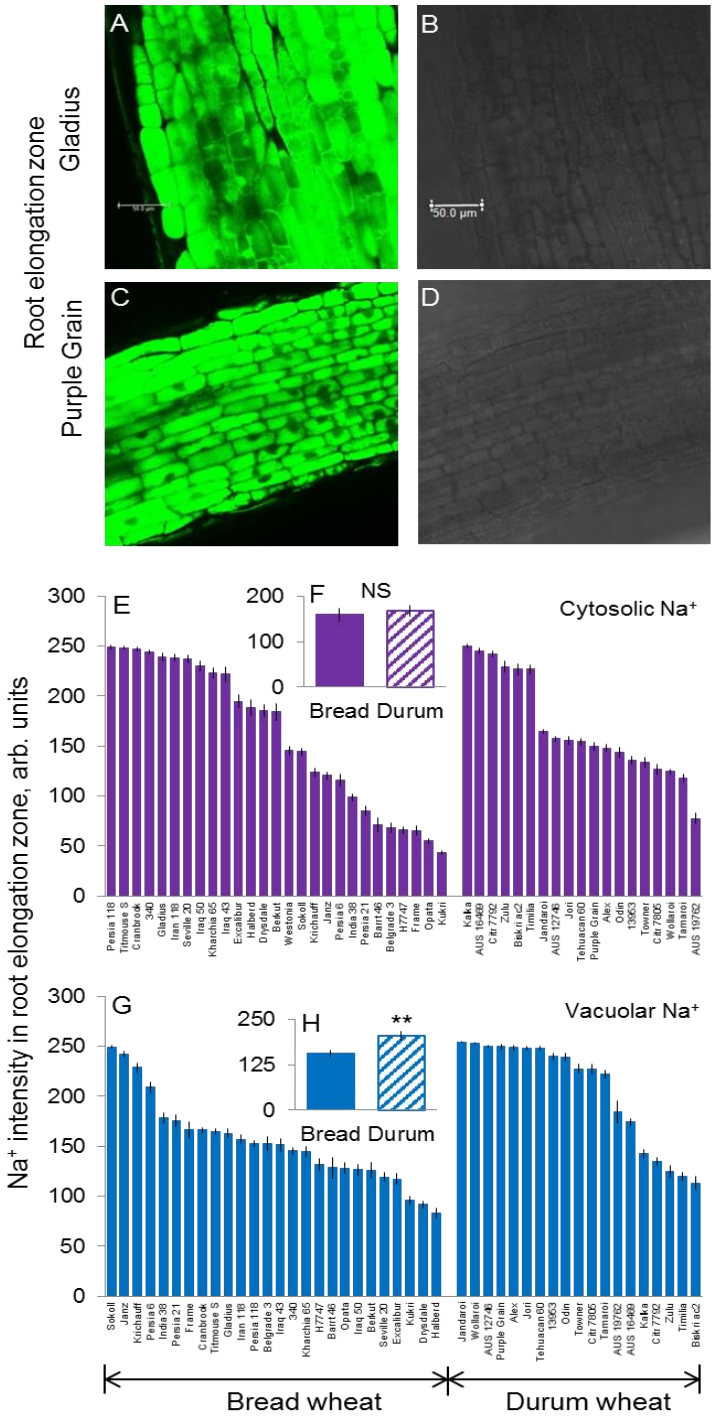


Fig. 4. Genetic variability of Na^+ intensity in the cytosol and vacuole in the root elongation zone in wheat. Representative CoroNa Green dye images in the root elongation zone in bread wheat Gladius (A) and durum wheat Purple Grain (C). B and D show the corresponding light images. Intensity of CoroNa Green fluorescence (arb. units) in the cytosol (E) and vacuole (G) in the root elongation zone of 46 wheat varieties contrasting in their salinity tolerance. Mean ± SE (n= 72 – 96 cells from at least 6 individual plants). Averaged pooled values for Na^+ intensity in the cytosol (F) and vacuole (H) in the root elongation zone for bread and durum wheat clusters. Mean ± SE (n= 19 (durum wheat) and 27 (bread wheat)).

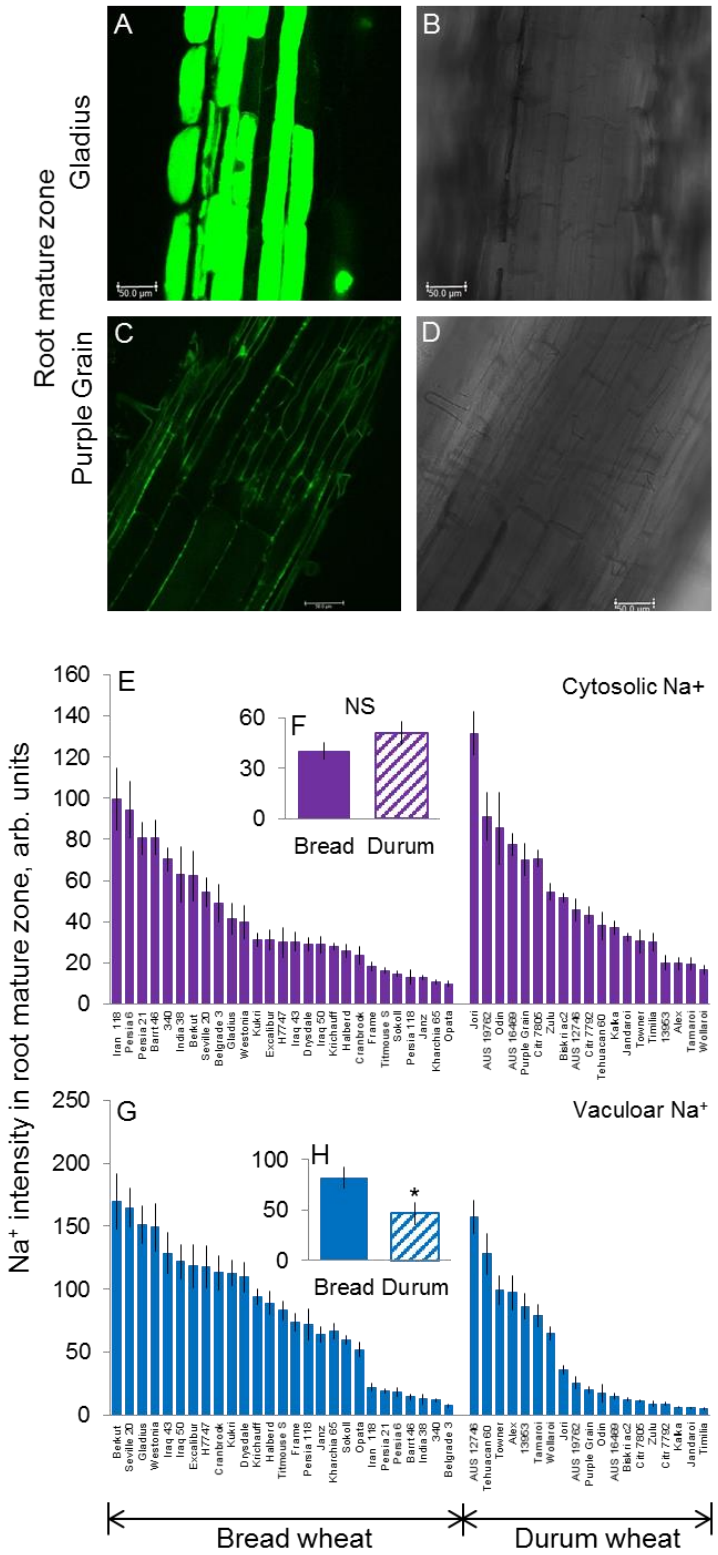


Fig. 5. Genetic variability of Na^+ intensity in the cytosol and vacuole in the root mature zone in wheat. Representative CoroNa Green dye images in the root mature zone in bread wheat Gladius (A) and durum wheat Purple Grain (C). B and D show the corresponding light images. Intensity of CoroNa Green fluorescence (arb. units) in the cytosol (E) and vacuole (G) in the root mature zone of 46 wheat varieties contrasting in their salinity tolerance. Mean ± SE (n= 72 – 96 cells from at least 6 individual plants). Averaged pooled values for Na^+ intensity in the cytosol (F) and vacuole (H) in the root mature zone for bread and durum wheat clusters. Mean ± SE (n= 19 (durum wheat) and 27 (bread wheat)).

9.3.5 Bread wheat possesses superior vacuolar Na^+ sequestration ability in the root mature zone

Laser confocal images of bread wheat Gladius and durum wheat Purple Grain were used to show Na^+ distribution between cytosol and vacuole in the root mature zone (Fig. 5A to D). A further investigation in 46 accessions of bread and durum wheat varieties was conducted. There was a large variability of Na^+ intensity in the cytosol (13.6 fold, Fig. 5E) and the vacuole (31.4 fold, Fig. 5G) found in screened varieties. Jori (durum wheat) showed the highest cytosolic Na^+ intensity 131.7 ± 10.4, whereas Opata (bread wheat) showed the lowest 9.7 ± 1.4 (Fig. 5E). Furthermore, the highest vacuolar Na^+ intensity was found in variety Berkut (bread wheat) 169.8 ± 21.8, compared with the lowest 5.4 ± 0.6 in the variety Timilia (durum wheat) (Fig. 5G). In the root mature zone, a significantly higher Na^+ intensity in the vacuole was found in bread than durum wheat (82.2 ± 9.9 vs 46.6 ± 10.9, $P < 0.05$; Fig. 5H), compared with no significant difference between bread than durum wheat in the cytosol (40.4 ± 5.1 vs 50.9 ± 7.0, $P > 0.05$; Fig 5F). Furthermore, overall salt tolerance in wheat was significantly negatively correlated ($r = 0.30$, $P < 0.05$; Fig. 6G) with cytosolic Na^+ intensity in the root mature zone but significantly positively correlated ($r = 0.44$, $P < 0.01$; Fig. 6H) with vacuolar Na^+ intensity in the root mature zone.

The known information of unidirectional Na^+ influx at whole root level, Na^+ extrusion and Na^+ distribution in cell compartments in different root zones is summarized in (Fig. 7A). Interestingly, a differential profile of average cytosolic and vacuolar Na^+ intensity was shown in different root zones cells. An increasing trend of both cytosolic and vacuolar Na^+ intensity was found in the root apex in both bread and durum wheat with the order: root meristem < transition < elongation zone; while a similar level of cytosolic and vacuolar Na^+ intensity was found between the root mature and meristem zone (Fig. 7B, C).

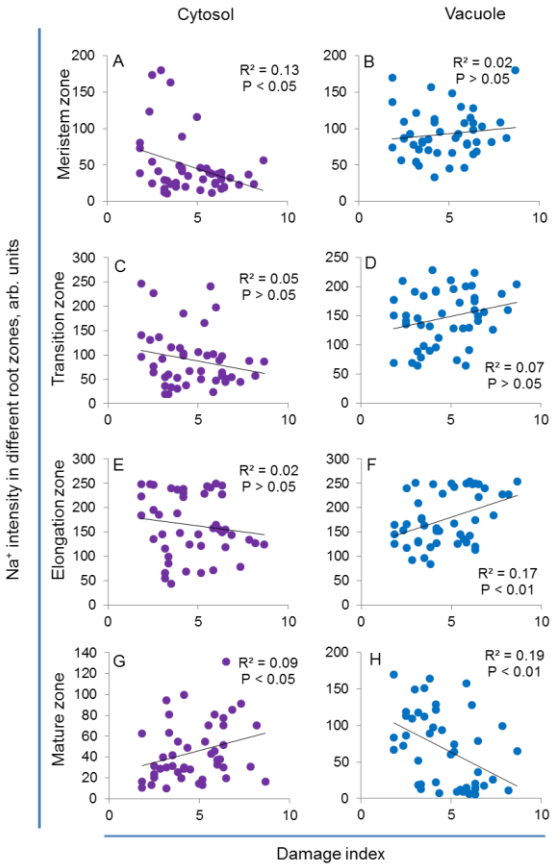


Fig. 6. Correlation between Na^+ distribution in the cytosol and vacuole in different root zones and overall salt tolerance in wheat. Correlation between overall salt tolerance and Na^+ intensity in the cytosol (A-meristem, C- ransition, E-elongation, G- mature zone) and vacuole (B-meristem, D-transition, F- elongation, H-mature zone). Each point represents a separate variety.

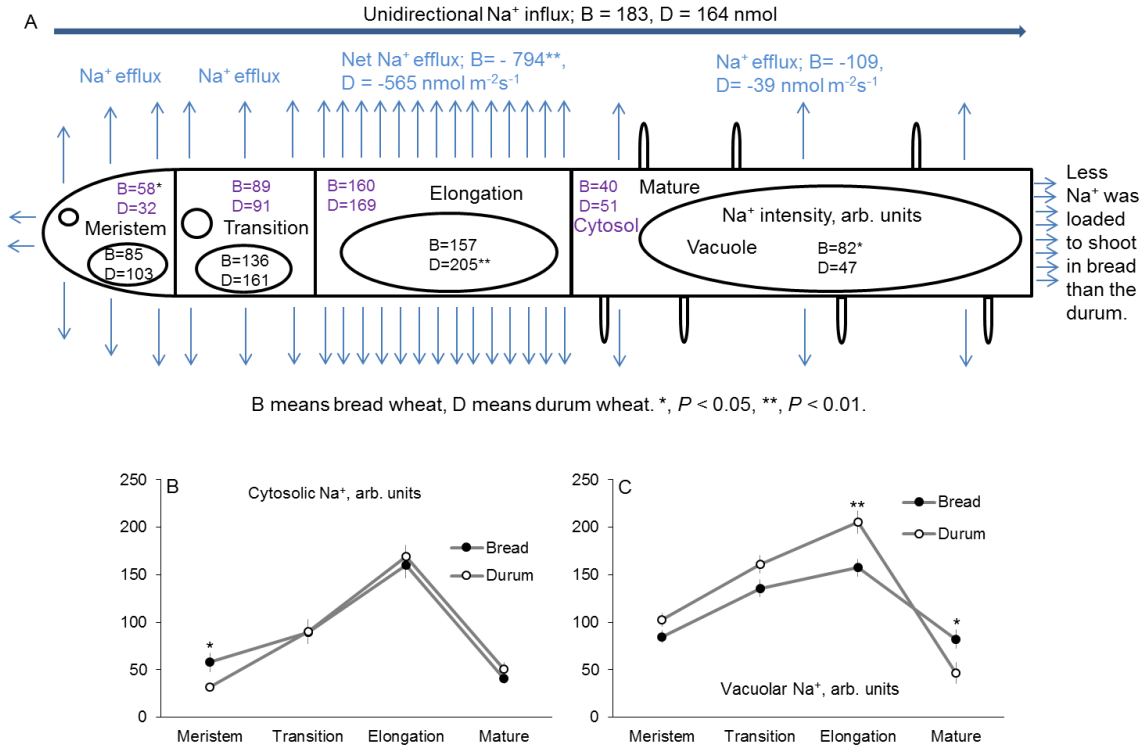


Fig. 7. Proposed model showing differential patterns of Na^+ distribution in cell compartments and Na^+ efflux from cytosol in root zones.

9.3.6 Plant performance after removal of the root meristem zone

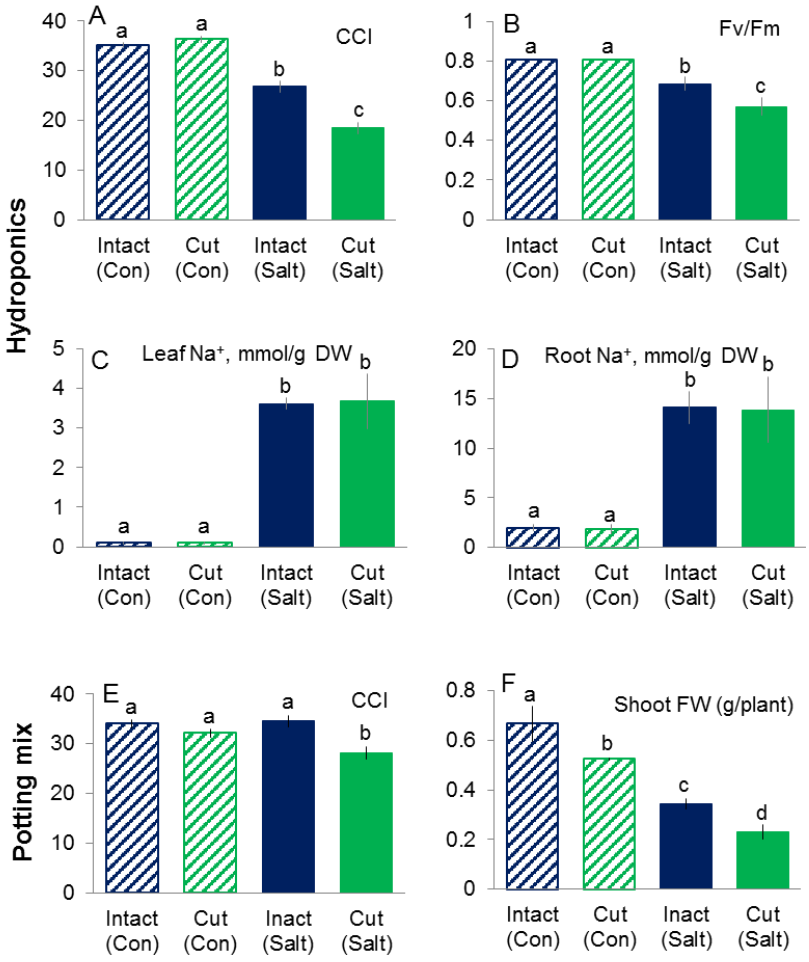


Fig. 8. Performance of plants with and without the removal of the root meristem zone under salt stress. A-D, CCI (A), F_v/F_m (B), leaf Na^+ content (C) and root Na^+ content (D) in intact plants (with intact roots) and cut plants (with the removal of the roots meristem zone) in the presence and absence of 200 mM NaCl in hydroponics. Mean ± SE (n = 24 − 36). E and F, CCI (E) and shoot FW (F) in intact plants and cut plants in the presence and absence of 200 mM NaCl in potting mix. Mean ± SE (n = 12 − 19). Salt tolerant bread wheat variety Kharchia 65 was used.

While there was no significant difference under non-saline conditions, significantly higher CCI (Fig. 8A) and F_v/F_m (Fig. 8B) values were found in intact plants compared to cut plants under salt stress (in the hydroponic experiments). Interestingly, there was no difference in Na^+ content in either leaf or root between intact and cut plants (Fig. 8C, D). It is suggested that either the distribution of Na^+ between cytosol and vacuole in mesophyll cells might be different between intact plants and plants with removed root meristem zone, or the root meristem zone might execute the role of a salt sensor by

sensing osmotic stress or integrating plant hormones. Similar to the results in the hydroponic experiment, significantly higher CCI values were found in intact plants (variety Kharchia 65) than in cut plants grown in potting mix (Fig. 8E) under salt stress conditions. Shoot FW was also significantly higher in intact plants (Fig. 8F). This validates our finding that leaf chlorophyll content in salinized plants was affected by removal of root meristem zone, suggesting that removal of the root meristem zone affects Na^+ distribution in leaf cells under salt stress. At the same time no improvement of salt stress performance was observed in the presence of plant hormone kinetin in both intact and cut plants (data not shown).

9.3.7 Relative expression level of *TaNHX1*, *TaVP*, *TaSOS1*, and *TaHA1* genes

Although gene expression was significantly supressed under salt stress, plants with intact roots showed 3.1- and 5.6-fold higher relative expression level of *TaNHX1* (Fig. 9A) and *TaVP* (Fig. 9B), respectively, than plants with a removed root meristem zone. No significant difference was found under non-saline condition between expression levels of *TaNHX1* nor *TaVP*. Unlike *TaNHX1*and *TaVP*, there were no differences between intact and cut plants under salt stress in relative expression levels of *TaSOS1* (Fig. 9C) and *TaHA1* (Fig. 9D), whereas transcript levels of *TaSOS1* and *TaHA1* in cut plants were significantly higher compared with intact plants under non-saline condition.

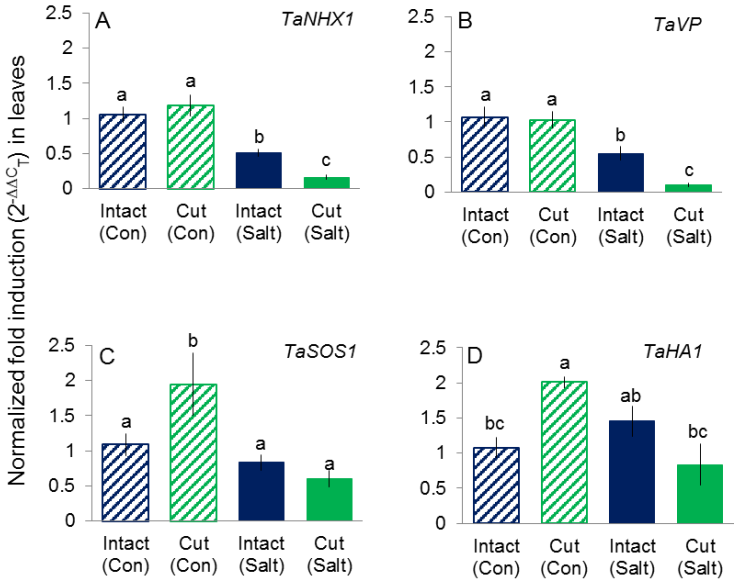


Fig. 9. Relative expression levels of genes in seedlings with and without the removal of the roots meristem zone under salt stress. Relative expression level of *TaNHX1* (A), *TaVP* (B), *TaSOS1* (C), and *TaHA1* (D) in intact plants (with intact roots) and cut plants (with the removal of roots meristem zone) in the presence and absence of 200 mM NaCl. Salt tolerant bread wheat variety Kharchia 65 was used. Mean ± SE (n = 8 − 10).

9.4 Discussion

9.4.1 Na^+ extrusion in the root elongation zone but not the root mature zone correlates with the overall salt tolerance in wheat

Na^+/K^+ ATPase expels three Na^+ ions from the cell in exchange for two K^+ ions in animal cells (Yatime et al., 2011; Galva et al., 2012). In lower plants e.g. *Physcomitrella patens* exclusion of Na^+ can be achieved by sodium ATPase (PpENA1) (Benito and Rodríguez-Navarro, 2003; Lunde et al., 2007). Until now, SOS1 Na^+/H^+ antiporter has been reported as the only pathway to exclude Na^+ from the cytosol to the apoplast in higher plants. GUS expression analysis showed that SOS1 was preferentially expressed in the root apex but not the mature zone in *Arabidopsis* (Shi et al. 2002). This was confirmed by our functional studies on wheat. While in the mature root zone Na^+ efflux ranged between -20 to -70 $nmolm^{-2}s^{-1}$ (Cuin et al. 2011), over 10-fold higher Na^+ extrusion ability was observed in the root elongation zone, ranging from -284 ± 39 to -1584 ± 237 nmol m^{-2} s^{-1} (Fig. 1). Also, in the present study, we found that bread wheat possesses significant higher Na^+ extrusion ability in the root elongation zone than durum wheat, and this Na^+ extrusion ability in the root elongation zone is strongly and positively correlated with overall salt tolerance in wheat (Fig. 1). Taken together, these results suggest that Na^+ extrusion in the root elongation zone but not root mature zone is correlated with the overall salt tolerance in wheat.

Wheat is a salt excluder (Kingsbury et al., 1984; Cuin et al., 2008; He et al., 2010), and the Na^+ exclusion trait plays an important role in its overall salt tolerance (James et al., 2011; Munns et al., 2012). In durum wheat, low Na^+ genotypes showed significant less decline in leaf chlorophyll content and thus better salt tolerance than the high Na^+ ones under moderate salt stress (Husain et al., 2003). Moreover, compared with a strong and positive correlation found in wheat (Fig. 1), no significant correlation between root Na^+ extrusion in the root elongation zone and overall salt tolerance was found in barley (Chapter 8), suggesting root Na^+ extrusion is important for salt excluder species e.g. wheat, but no salt includer species e.g. barley.

9.4.2 Tissue specificity of Na^+ distribution in different root zones in the context of overall salt tolerance in wheat

More and more attention has recently been paid to the study of tissue specificity of salinity stress e.g. salt stress regulator genes (Dinneny et al., 2008) and ion distributions in different cell types (Lauchli et al., 2008) or different zones (Wu et al., 2015). In the present study, we extended our previous finding (Wu et al., 2015) of the tissue specificity of vacuolar Na^+ sequestration in different root zones in a small number of bread wheat cultivars to a large number of bread and durum wheat accessions. Similar to previous

results (Wu et al., 2015), the overall salt tolerance was strongly and positively correlated with vacuolar Na^+ sequestration in the root mature zone but not the root apex (Fig. 6), suggesting the importance of vacuolar Na^+ sequestration ability in the root mature zone in overall salt tolerance at least in wheat. This is a reasonable strategy for plants since the majority of roots are in the root mature zone.

Furthermore, as summarized in Fig. 7, the trend of Na^+ intensity in both the cytosol and vacuole in roots of bread and durum wheat was shown as: root elongation > transition > meristem zone ≈ mature zone. What is the physiological rationale behind the maintenance of different cytosolic Na^+ levels in different root zones under salt stress? Does it originate from the inability of specific root cells to exclude Na^+ from the cytosol, or is it needed for salt stress sensing and signal transduction? Can we talk about possible "Na^+ signatures" in this case, similar to those for Ca^{2+} (Dodd et al., 2010; Kudla et al., 2012) and ROS (Mittler et al., 2011; Suzuki et al., 2012)? Another possibility for the differential cytosolic Na^+ accumulation in root zones is that they might refer to different osmotic pressure, and thus it can differentially regulate the mechanosesitive channels (Hamilton et al., 2015) and thus Ca^{2+} transport (Kurusu et al., 2013) in different root zones. The role of root water status derived hydraulic signals in long distance signalling was recently discussed (Christmann et al., 2013).

9.4.3 Leaf Na^+ distribution might be affected by removal of root meristem zone

The root meristem zone is known to be important for postembryonic root growth. Under salt stress, the structure of meristematic cells of barley roots was changed and an increase in vacuolation was found (Huang and van Steveninck 1990). Salt stress induced nuclear and DNA degradation and PCD phenotypes in root meristematic cells was also found in soybean (Liu et al., 2000) and Arabidopsis (Huh et al., 2002), respectively. Further, the mitotic index of root meristem cells was found to be significantly reduced under salt stress (Shakirova et al., 2003). In the present study, bread wheat shows significant higher cytosolic Na^+ intensity in the root meristem zone than durum wheat although no significant difference in vacuolar Na^+ intensity in the root meristem zone was found (Fig. 2), suggesting the possible role of the root meristem zone in salt stress sensing.

At the cellular level, the Na^+ distribution in cell compartments is mainly controlled by the rate of Na^+ entry (mediated by NSCC channels (Demidchik and Tester, 2002; Apse and Blumwald, 2007) and HKT transporters (Apse and Blumwald, 2007; Plett and

Møller, 2010)), Na^+ extrusion (mediated by SOS1 Na^+/H^+ antiporter, Shi et al., 2002), and vacuolar Na^+ sequestration (mediated by NHX1 Na^+, K^+/H^+ exchanger, Apse et al., 1999). In our study, a clear difference in chlorophyll content was observed between intact plants (with intact roots) and cut plants (with removal of roots meristem zone) under salt stress (Suppl. Fig. S4). Also, the cut plants showed significantly decreased F_v/F_m compared with intact plants under salt stress. In the hydroponics experiment, no difference in root FW was found between intact plants and cut plants under both control and salt stress conditions (data not shown). It is suggested that the significantly decreased CCI and Fv/Fm in cut plants than intact plants under salt stress are most likely due to the effect of removal of the proposed salt sensor root meristem zone, and is not confounded by the possible decrease of sink strength of the roots which might in turn have negative feedback effects on photosynthesis and translocation of assimilates.

Furthermore, we found that although it is suppressed under salt stress, the transcript level of *TaNHX1*and *TaVP* was a few fold ($P < 0.01$) higher in intact plants than in cut plants whereas no significant difference of transcript level of TaSOS1 was found (Fig. 9). NHX1 Na^+, K^+/H^+ exchanger is known to be fueled mainly by the vacuolar PPase (Silva and Gerós, 2009). It suggests that removal of the root meristem zone affected the ability of vacuolar Na^+ sequestration, resulting in the accumulation of more Na^+ in cytosol and thus worse photosynthetic ability in the cut plants than the intact plants. How this long distance regulation was achieved is still unknown. It is known that cytokinin and auxin antagonistic interaction is important in controlling meristem activity (Ioio et al., 2007, 2008). Thus, the change at the hormonal level might be one explanation of the altered Na^+ distribution in salinized plants after removal of the root meristem zone. Taken together, our results suggest that the root meristem zone is involved in salt stress sensing and long distance salt stress signal transduction, at least in bread wheat.

Suppl. Fig. 4. Leaf chlorosis in salinized plants with and without the removal of roots meristem zone under salt stress. More severe leaf chlorosis was observed in cut plants (with the removal of roots meristem zone) than intact plants (with intact roots) after 10 days salt stress (200 mM NaCl, hydroponics).

9.4.4 Long distance signal transduction in plant salt stress sensing

Despite some recent reviews (Maathuis, 2014; Shabala et al., 2015a, b), exactly how plants sense and convey salt stress signals is still obscure, especially long distance signal transduction e.g. from root to shoot. Here, our results suggested that leaf Na^+ distribution in salinized plants might be affected by the removal of the root meristem zone (Fig. 8), suggesting that some signal produced in the root meristem is transmitted to the shoot under stress conditions, and that coordinating with this signalling results in altered plant phenotype and compromised plant Na^+ sequestration ability in the shoot. The nature of this signal remains unexplored. Root derived phytohormones and/or ROS are amongst the likely candidates. Indeed, after the removal of the root meristem zone (disruption of the apical dominance), branch roots appeared 5 days later in plants grown in ¼ Hoagland solution but not in plants grown under saline condition (Suppl. Fig. 5), suggesting plant hormones e.g. cytokinins might be involved in shaping/regulation of the root architecture under salt stress. However, exogenous application of kinetin (one of the forms of cytokinins) to plants with removed root meristem did not result in an improvement of plant performance under salt stress (data no shown). This suggests that cytokinins are not likely to be the signalling molecule in question. Other candidates such as brassinosteroid (Hacham et al., 2011), ROS (Gilroy et al., 2014), Ca^{2+} (Dodd et al., 2010), ABA (Liang et al., 2007), and sugars (Rosa et al., 2009, Krasensky and Jonak, 2012) participate in the long distance salt stress signal transduction and thus should be considered.

Suppl. Fig. 5. Branch roots appeared in cut plant (with the removal of roots meristem zone) grown in ¼ Hoagland solution.

ROS was previously suggested to play a role in a systematic signal transduction under abiotic stress (Miller et al., 2009, Baxer et al., 2014). However, its role in the long distance salt stress signal transduction still needs to be studied. If ROS are indeed

involved, how do they communicate with other cells and relay the salt stress signal from root to shoot? How does this signaling affect the process of controlling leaf Na^+ distribution in salinized plants after removal of the root meristem zone? Besides plant hormones and ROS, other signalling molecules e.g. Ca^{2+} and sugar might be also participated in regulating leaf Na^+ distribution in salinized plants after removal of the root meristem zone. Ca^{2+} is known as a second messenger and is involved in a broad range of plant cell activities in regulating plant growth and development (Hepler, 2005). Recently, salt stress induced Ca^{2+} waves were found to be associated with rapid, long distance root to shoot signalling in plants (Choi et al., 2014). Furthermore, overexpression of ABF2 (ABRE-binding bZIP factor), an essential component of glucose signalling, improved salt stress tolerance in Arabidopsis (Kim et al., 2004). Taken together, the signalling events involved in this phenomenon may be a complex coordinated by different signalling molecules in a "fine tune" and/or "coarse tune" mode. Future studies should be conducted to investigate the identity of specific signalling molecules and their role in the phenomenon that leaf Na^+ distribution in salinized plants was affected by the removal of its root meristem zone.

Chapter 10

General discussion

Improving the salt tolerance of crops is important to secure food supply in the future, in light of a decline in crop yield due to raising salinity issues and increasing world population. Until now, phenotype selection, QTL/marker assisted selection, and genetic engineering have been the main pathways used to produce salt tolerant crops. Two basic genetic approaches that are currently being used to improve stress tolerance include: (1) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci and subsequent marker-assisted selection, and (2) generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance (Yamaguchi and Blumwald, 2005). The development of high-density DNA maps that incorporate microsatellite markers, RFLP (restriction fragment-length polymorphisms) and AFLP (amplified fragment-length polymorphisms), and advances in marker-assisted selection techniques will facilitate pyramiding traits of interest to attain substantial improvement in crop salt tolerance (Yamaguchi and Blumwald, 2005). Moreover, two additional approaches that are based on population genetics and that make use of genetic diversity have emerged: association mapping and selection screens (Takeda and Matsuoka, 2008). The advantages and disadvantages of the above described approaches are given in Table 1.

Table 1 The advantages and disadvantages of the approaches used in breeding program.

Breeding methods	Advantages	Disadvantages
Traditional selection	accepted by the public as "natural", safety	time consuming; affected by environmental factors
QTL/marker-assisted selection	time efficient; the impact of environmental effects on the traits is reduced (Munns and James, 2003; Yamaguchi and Blumwald, 2005)	development of robust markers can be quite difficult, and is dependent on an accurate phenotype screen (Munns et al., 2002)
Gene chip/microarray	time efficient	high cost
Transgenic plants	overcomes the limitation of crossing between species	poor public acceptance

Although several salt-tolerant plant genotypes have been developed through transgenic approaches, they have often failed or exhibited limited success under field saline conditions. Plant growth and development under saline conditions in the field are often influenced by the cumulative effects of multiple environmental stresses and genetic factors, which may not have been considered during the development of salt-tolerant transgenic plants (Jamil et al., 2011). While the use of *Arabidopsis* as a model species provided some clues about the enhanced stress tolerance based on individual genes in a number of pathways, genes with a stress-alleviating quality under controlled conditions have failed to generate stress protection in the glasshouse or field (Yang et al., 2009; Wu et al., 2012; Adem et al., 2015). Adoption of inappropriate screening techniques or selection criteria may also lead to selection of genotypes that may not be stress tolerant in field conditions (Jamil et al., 2011).

To better understand salinity tolerance in plants and use it for breeding robust salt tolerant crops, more studies are required that focus on specific crops and that do not rely on the results from *Arabidopsis* since this model plant is salt sensitive. While the use of *Arabidopsis thaliana* as a genetic model plant facilitated the elucidation of several Na^+ transport processes that underlie salinity tolerance (Møller and Tester, 2007). Wu et al. (2012) recently sequenced the genome of a halophyte *Thellungiella salsuginea*, a close relative of *Arabidopsis*, which has exceptionally high resistance to cold, drought, and oxidative stresses as well as salinity. *T. salsuginea* is exemplary as it has a short life cycle, self-fertility, is genetically transformable, has a larger relative genome size (approximately twice that of *A. thaliana*) and there are ecotypes available that show a range of stress responses (Wu et al., 2012). Learning from halophytes (Shabala and Mackay, 2011; Shabala et al., 2014) can be another approach for improving salt tolerance in some important crops e.g. wheat and barley.

Wheat and barley are two major crops cultivated world-wide; both of them experience large yield penalties from salinity in their production habitats. However, the complexity of salinity stress tolerance impedes the progress of breeding robust salt tolerant crop species. With no truly salt tolerant barley and wheat genotypes available, the aims of this Grain Research & Development Corporation funded research were: 1) to investigate the importance of the ability of mesophyll cells to retain K^+ in cytosol in the overall salt tolerance in wheat and barley, as well as the molecular and physiological identity of the ion channels involved; 2) to investigate the role of leaf tissue tolerance in the overall salt tolerance in wheat and barley, as well as its relative contribution in each species; 3) to

investigate the role of the tissue specific Na^+ distribution in cell compartments in different root zones, as well as its function in salt stress signalling and adaptation; 4) to quantify the relative contribution of root cytosolic Na^+ extrusion and K^+ retention, and vacuolar Na^+ sequestration in the overall salt tolerance in wheat and barley at the tissue specific level; 5) to develop screening methods to quickly screen salt tolerance traits to benefit breeding programs.

The major findings in this work were as follows.

Barley:

(1) The ability of leaf mesophyll cells to retain K^+ under saline conditions is correlated with the overall salt tolerance. This trait is mediated by regulation of depolarization activated KOR channels. (2) Tissue salinity tolerance is arguably the most essential trait conferring the overall salt tolerance in this species. (3) Both root Na^+ extrusion and cytosolic K^+ retention in the root elongation zone play only a minor role in salt tolerance in barley. (4) While the depolarization activated KOR channels mediated NaCl-induced K^+ efflux in the *mature* root zone, these were voltage independent NSCC channels that played the main role in controlling NaCl-induced K^+ efflux in the *elongation* root zone. (5) Vacuolar Na^+ sequestration in the elongation zone correlated strongly with the overall salt tolerance. (6) Stress-induced increase in the transcript levels of both *HvNHX1* and *HvVP1* was not sufficient to achieve efficient vacuolar Na^+ sequestration, the involvement of FV/SV vacuolar Na^+/K^+ permeable channels is also required.

Wheat:

(1) The ability to maintain K^+ in leaf mesophyll cells under salt stress is correlated with the overall salt tolerance. (2) This trait is mediated by regulation of NSCC channels (activated by ROS generated in chloroplasts, see details in Appendix 2). (3) Durum wheat possessed higher salt tissue tolerance, presumably as a compensation for the inability of this species to exclude Na^+ from uptake. (4) Vacuolar Na^+ sequestration correlated with the overall salt tolerance in the root mature zone but not in the root apex in wheat. (5) Significantly higher cytosolic Na^+ intensity was found in salt tolerant rather than sensitive group in the root meristem zone, suggesting the possible role of meristem as a salt sensor. (6) Leaf Na^+ distribution and relative gene expression of *TaNHX1* and *TaVP1* in leaves was affected by the removal of the root meristem zone, further emphasising the importance of meristematic cells in salt stress sensing and long distance salt stress signal transduction from the root to the shoot.

The present thesis confirmed the complexity of salinity stress tolerance mechanisms in plants, and demonstrated the importance of investigating Na^+ extrusion, K^+ retention and vacuolar Na^+ sequestration at the tissue-specific level. Further insights into this issue can give a detailed map for molecular breeders to enable them to target the precise mechanism/s at the cell- and tissue- specific level. Also, possible confounding effects e.g. breeding plots being used for multiple purposes induced by inappropriate molecular breeding might be eliminated.

Despite some recent successes (e.g. Munns et al., 2012), the progress in plant breeding for salinity stress tolerance is still not as advanced as one would expect. Compared with non-saline conditions, wheat plants overexpressing *HKT* (high affinity K^+ transporter) (James et al., 2002; Munns et al., 2012) or *NHX1* (Xue et al., 2004) genes still show significant yield penalties, although they perform better than non-transformed controls. It is possible that some of the traits targeted by breeders were not compatible. As we showed in the present thesis, plant salt tolerance involves complex (interacting?) mechanisms that are highly tissue specific and it is argued that salinity stress tolerance may be achieved only via the pyramiding approach (Flowers and Yeo, 1995; Shabala, 2013). In addition to the physiological and genetic complexity of the salinity tolerance trait, the lack of convenient screening techniques also impedes the progress in breeding. Although agronomical (e.g. biomass/yield, plant survival, or leaf injury; Munns and James, 2003; Colmer et al., 2005) and biochemical (e.g. antioxidant activity or compatible solutes content; Ashraf and Harris, 2004) markers are showed to be convenient as rapid screening tools, they fail to account for the tissue-specificity of physiological mechanisms conferring salinity tolerance.

Previous extensive studies have shown that non-invasive microelectrode ion flux estimation (MIFE) (see Newman et al. 1987; Newman 2001 for theory and Chen et al., 2007b; Wu et al., 2013 for the practical use) is effective for cell-based screening of a large number of genotypes with specific salinity tolerance traits that are rapid, low cost and reliable. By using the MIFE technique, researchers can visualise the measured pattern and ion fluxes in real time with net ion fluxes being automatically recorded by computer. This technique fulfils the requirement of high throughput screening based on a specific salinity tolerance trait and also can be used in investigating salinity tolerance mechanisms. After the rapid MIFE screening, elite varieties can be distinguished based on a specific physiological trait that refers to salinity tolerance and these varieties can be recommended to plant breeders as the salt tolerant gene donors. This approach will be

time-saving and benefit the breeding programme for salt tolerant crops. Hence, combining MIFE technique with QTL/marker assisted selection may become a powerful way to breed real salt tolerant crops in field.

Some suggestions and potential protocols originated from this thesis are given below:

1) An inexpensive and rapid high throughput method to screen for salt tolerance was developed by evaluating the plants ability to maintain K^+ in leaf mesophyll using the MIFE technique. The next logical step would be to undertake QTL mapping of genes conferring the mesophyll K^+ retention trait. Recommended DH parents to target the mesophyll K^+ retention trait in barley and wheat are given in Table 2.

2) By screening the chlorophyll content change in salt treated excised leaves, the leaf tissue specific salinity stress tolerance can be rapidly assessed. However, one should note here that, in contrast to the positive correlation in barley, leaf tissue specific salinity stress tolerance is negatively correlated in wheat and thus works as a compensation mechanism. Our research suggests that targeting vacuolar Na^+ sequestration to improve the leaf tissue tolerance and pyramiding it with other salt tolerance mechanisms might be beneficial to the salt tolerant breeding program in barley. However, if researchers/breeders are targeting genes conferring leaf tissue specific salinity stress tolerance in wheat, then the salt-*sensitive* varieties should be used as gene donors for this trait. Recommended DH parents to target leaf tissue tolerance trait in barley and wheat are given in Table 2.

3) Using CoroNa Green fluorescent dye, we have found that vacuolar Na^+ sequestration in the barley root elongation zone and the wheat root mature zone were strongly correlated with the overall salt tolerance. This method allows screening 15 to 20 genotypes per day by using the current protocol (Wu et al., 2015a). Recommended DH parents to target better root vacuolar Na^+ sequestration traits in barley and wheat are given in Table 2.

4) Na^+ extrusion from the root elongation zone was strongly correlated with the overall salt tolerance in wheat. Recommended DH parents to target better root SOS1 Na^+ extrusion traits in wheat are given in Table 2.

Table 2 Best performing varieties (potential gene "donors") and recommended DH pairs for each trait.

Physiological trait targeted	**Best performing varieties**	**Recommended DH parents**
Barley		
High K^+ retention in mesophyll	YWHKSL, Numar, Yerong, YYXT, AC Burman	Numar & Aizao 3; YWHKSL & Keel
Better leaf tissue tolerance	Honen, SYR 01, ZUG293, AC Burman, Numar	Honen &Naso Nijo; SYR 01 & Aizao 3
Better root vacuolar Na^+ sequestration	ZUG 293, RGZLL, Yerong, CM72, Numar	ZUG 293 & YSM 3; RGZLL & Yan 90260
High SOS1 root Na^+-extrusion activity	Gebeina, YUQS, CM72, Yerong, Dash	Gebeina & DYSYH; CM 72 & ZUG 403
Bread Wheat		
High K^+ retention in mesophyll	Westonia, Persia 118, Gladius	Persia 118 & 340; Westonia & Sokoll
Better leaf tissue tolerance	Persia 6, Baart 46, Iran 118	Persia 6 &India 38; Baart 46 & Opata
Better root vacuolar Na^+ sequestration	Berkut, Gladius, Westonia	Westonia & Belgrade 3; Gladius & 340
High SOS1 root Na^+-extrusion activity	Titmouse S, Gladius, H7747	TitmouseS & Belgrade 3; Gladius & Sokoll
Durum wheat		
High K^+ retention in mesophyll	13953, Frame, Jandaroi	13953 & C250; Frame & Odin
Better leaf tissue tolerance	AUS 16469, Citr 7805, Towner	AUS 16469 & 13953; Citr 7805 & Alex
Better root vacuolar Na^+ sequestration	Tehuacan 60, Alex, 13953	Tehuacan 60 & Timilia; 13953 & Kalka
High SOS1 root Na^+-extrusion activity	Covelle, Frame, 13953	Covelle & Biskri ac2; Frame & Caparoi

Taken together, the results of this work discovered several previously unknown (unutilised) traits conferring salinity stress tolerance in barley and wheat. This knowledge, alongside with technological developments and suggested high throughput protocols, may be used by plant breeders in developing salt-tolerance barley and wheat germplasm to minimise salinity-induced yield losses.

This appendix has been removed for copyright or proprietary reasons.

Wu, H., Shabala, L., Zhou, M., Shabala, S., (2015). MIFE technique-based screening for mesophyll K+ retention for crop breeding for salinity tolerance, Bio-protocol 5(9), e1466, 1-10

Appendix 2

Chloroplast-generated ROS dominates NaCl-induced K^+ efflux in wheat leaf mesophyll[#]

Abstract

Mesophyll K^+ retention ability has been recently reported as an important component of salinity stress tolerance in wheat. In order to investigate the role of ROS in regulating NaCl-induced K^+ efflux in wheat leaf mesophyll, a series of pharmacological experiments was conducted using MV (methyl viologen, superoxide radical inducer), DPI (an inhibitor of NADPH oxidase), H2O2 (to mimic apoplastic ROS), and EGCG ((−)-Epigallocatechin gallate, ROS scavenger). Mesophyll pre-treatment with 10 μM MV resulted in a significantly higher NaCl-induced K^+ efflux in leaf mesophyll, while 50 μM EGCG pre-treatment alleviated K^+ leakage under salt stress. No significant change in NaCl-induced K^+ efflux in leaf mesophyll was found in specimens pre-treated by H2O2 and DPI, compared with the control. The highest NaCl-induced H^+ efflux in leaf mesophyll was also found in samples pre-treated with MV, suggesting a futile cycle between increased H^+-ATPase activity required and ROS-induced K^+ leak. Overall, it is suggested that, under saline stress, K^+ efflux from wheat mesophyll is mediated predominantly by non-selective cation channels (NSCC) regulated by ROS produced in chloroplasts, at least in bread wheat.

Keywords

Chloroplast, K^+ channels, K^+ retention, leaf mesophyll, ROS

#, This appendix has been published as: Wu H, Shabala L, Zhou M, Shabala S (2015) Plant Signaling & Behavior 10 (5): e1073793

Potassium is a critical nutrient in plant's life. It has a central role in the maintenance of key metabolic processes, and its deficiency in plants can result in significant suppression of their photosynthetic ability (Zhao et al., 2001; Cakmak, 2005; Anschütz, et al., 2014). To cope with low availability of K^+ in soil (0.1-1mM) (Wang and Wu, 2013), a sophisticated K^+ uptake system was evolved in plants to reach a high concentration of cytosolic K^+ (100 mM) (Wang and Wu, 2013; Dreyer and Uozumi, 2011) in plant cells. Besides root K^+ uptake, efficient K^+ retention was also believed to be involved in this process, since up to 80% K^+ in shoot may be recirculated back to the root (Marschner et al., 1996). Under stress conditions such as salinity, K^+ efflux is thermodynamically favoured (Shabala and Pottosin, 2014). Over the last decade, the importance of K^+ retention in the cytosol has emerged as an additional component in plant salinity tolerance mechanisms, both in root (Chen et al., 2005, 2007c; Cuin et al., 2008; Smethusrt et al., 2008; Sun et al., 2009) and leaf (Wu et al., 2013, 2014, 2015d) tissues in various species.

Earlier experiments have suggested that NaCl-induced K^+ efflux in leaf mesophyll is mediated mainly by two types of plasma membrane channels: KOR channels (K^+ outward rectifying channel) and NSCC (non-selective cation) channels (Shabala et al., 2006). KOR channels are always gated by membrane depolarization (Véry and Sentenac, 2003; Wang and Wu, 2013), and their activity is increased in the presence of ROS (i.e. $OH^{\bullet}$) (Demidchik et al., 2010). In their turn, some of NSCC can also be activated by various ROS species such as H_2O_2 or $OH^{\bullet}$, as revealed in direct patch -clamp experiments (Demidchik et al., 2003, 2007; Zepeda-Jazo et al., 2011). Given the prominent role ROS production plays in salt-stress responses (Gill and Tuteja, 2011), it is therefore important to understand which of the channels plays a major role in cytosolic K^+ retention under stress conditions. In our preliminary paper (Wu et al., 2014), we showed that NSCC but not KOR channels dominated NaCl-induced K^+ efflux from wheat leaf mesophyll. We also found no significant effects of DPI (an inhibitor of NADPH oxidase) pre-treatment on NaCl-induced K^+ efflux from leaf mesophyll, suggesting that ROS produced by NADPH oxidase was not the main source in regulation of the NaCl-induced K^+ efflux in wheat leaf mesophyll. The aim of current work was to reveal the identity and source of ROS signals mediating K^+ efflux from leaf mesophyll under saline conditions.

Many sources of intracellular ROS production were characterized in plants. This includes ROS production by the plasma membrane located NADPH oxidase system, peroxisomes, chloroplast, mitochondria, vacuole, and apoplast (Demidchik et al., 2014, Bose et al., 2014a). While in animal systems mitochondria are the main sources of ROS

production, chloroplasts and peroxisomes are the largest ROS producers in green plant tissues, producing 20-fold more ROS than mitochondria under the light (Wrzaczek et al., 2013). With this notion, internal H_2O_2 concentration in leaf mitochondria remained unchanged under salt stress in both salt-tolerant and salt-sensitive pea varieties, compared with the control (Hernández et al., 1993). Importantly, chloroplasts not only provide cell with the energy but also represent a sensor of environmental information, and chloroplast redox signals help to acclimate the organism to environmental stresses (Laoli et al., 2014).

In the present paper, NaCl-induced K^+ efflux in leaf mesophyll was investigated in samples pre-treated with methyl viologen (MV, a redox-active compound that generates superoxide anions in chloroplasts, Fujibe et al., 2004), H_2O_2 (mimicking apoplastic ROS), and EGCG ((−)-Epigallocatechin gallate, a ROS scavenger; Cos et al., 1998; Nakagawa and Yokozawa, 2002), by using MIFE technique (Fig. 1; see also Wu et al., 2014 for details).

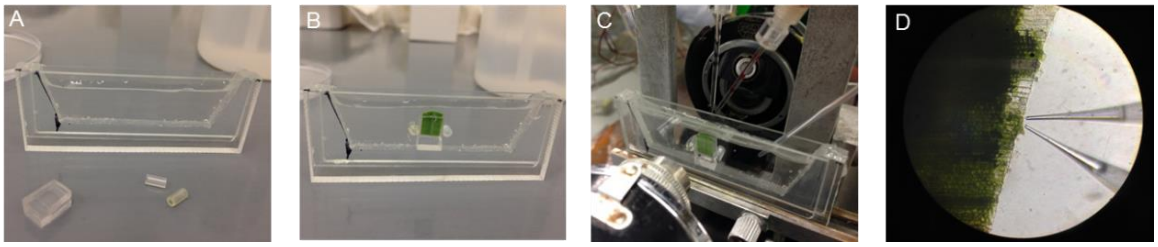


Fig. 1 – Logistics of ion flux measurements using the MIFE technique. (A), Measuring chamber, leaf segments holder, and the plastic stopper used for immobilisation; (B), leaf segments immobilized in the measuring chamber; (C), the measuring chamber with immobilized specimen assembled on the cartridge of hydraulic manipulator; (D), the tips of K^+ and H^+ ion selective microelectrodes are aligned next to the surface of exposed leaf mesophyll. See also Wu et al. 2014 for details.

MV and H_2O_2 are widely used in plant research for oxidative stress. EGCG is a well-known ROS scavenger that protects lipids and membrane proteins from oxidative damage (Hernández et al., 2009; Saffari et al., 2004). It is widely used in animal research (Johnson and Loo, 2000; Jung et al., 2007; Sugisawa and Umegaki, 2002; Zhang et al., 2000), and was also applied to plant species by several authors (Lee et al., 2008; Hernández et al., 2006). As showed in Fig. 2, specimens pre-treated with MV showed significantly higher NaCl-induced peak K^+ efflux from leaf mesophyll as compared with non-treated control. The opposite trend was observed in EGCG pre-treated samples, where significantly lower NaCl-induced peak K^+ efflux was detected (Fig. 2). No significant difference in NaCl-induced K^+ efflux in leaf mesophyll was found between control and H_2O_2 pre-treated samples (Fig. 2a, b). It is suggested that compared with

apoplast and plasma membrane-located NADPH oxidase, the ROS generated by chloroplasts play the main role in regulating NaCl-induced K^+ efflux in leaf mesophyll, at least in wheat. Consistent with K^+ data, the highest NaCl-induced H^+ efflux in leaf mesophyll was found in specimens pre-treated with MV, showing over 10-fold higher H^+ efflux than in the control (Fig. 3). These results suggest that, in an effort to retain high cytosolic K^+ levels under MV-induced K^+ leak, plants are pumping out more H^+ to maintain membrane potential to take up external K^+ via inward-rectifying (KIR) channels. Yet, being unable to control MV-induced K^+ leak via NSCC this pumping might result in a futile cycle, potentially leading to a substantial waste of the ATP pool. Further studies are required to directly prove this model and understand a causal link between mitochondria-produced superoxide anions and NSCC activation in leaf mesophyll, in order to confer salinity stress tolerance in plants, that is directly proportional to plant's ability to retain K^+ in leaf mesophyll (Anschütz et al., 2014; Shabala and Pottosin, 2014).

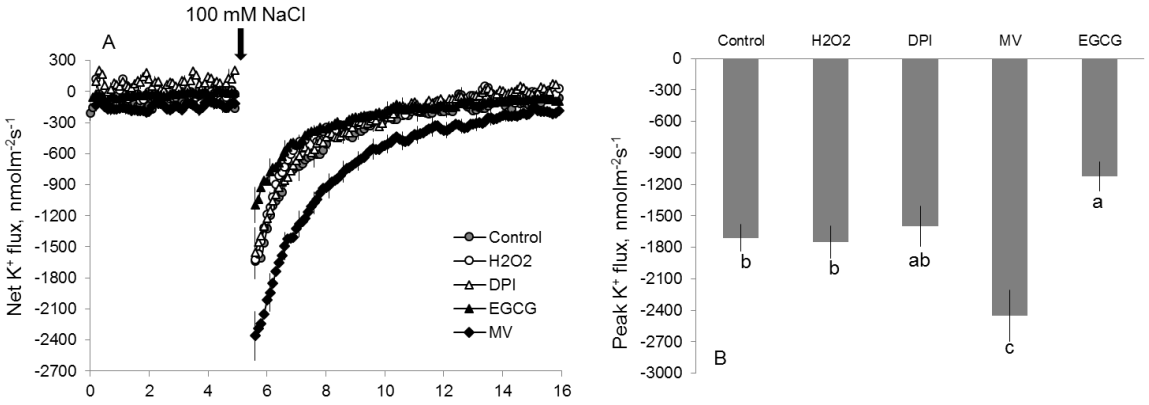


Fig. 2 – Kinetics of NaCl-induced K^+ efflux in leaf mesophyll (seven to 10 days old leaves of bread wheat seedlings used, cultivar Janz) pre-treated with different chemicals (A), peak K^+ efflux values from mesophyll samples exposed to 1 mM H_2O_2, 20 μM DPI, 10 μM MV, and 50 μM EGCG pre-treatments (1h) (B). Mean ± SE (n = 5 - 10). Different lower case letters represent significant difference ($P < 0.05$; oneway ANOVA based on Duncan's multiple range test, SPSS 20.0).

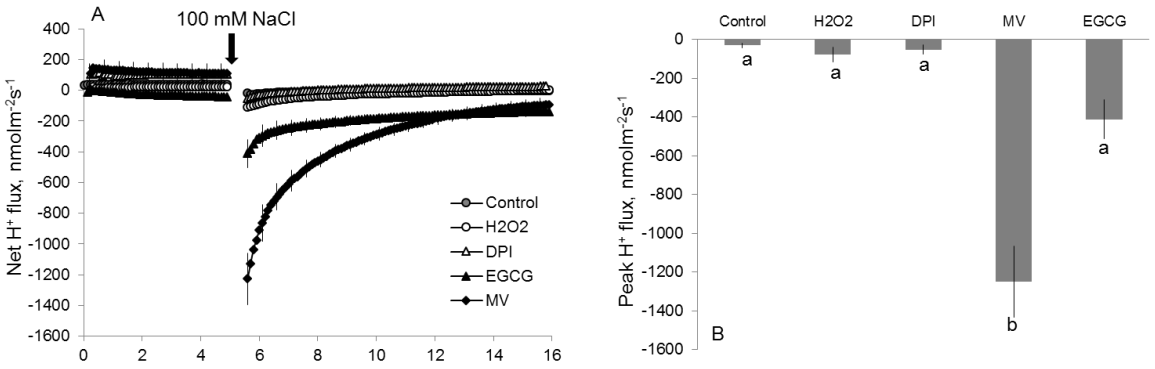


Fig. 3 − Kinetics of NaCl-induced H^+ efflux in leaf mesophyll (seven to 10 days old leaves of bread wheat seedlings used, cultivar Janz) pre-treated with different chemicals (A), peak H^+ efflux values from mesophyll samples exposed to 1 mM H_2O_2, 20 μM DPI, 10 μM MV, and 50 μM EGCG pre-treatments (1h) (B). Mean ± SE (n = 5 - 10). Different lower case letters represent significant difference ($P < 0.05$; oneway ANOVA based on Duncan's multiple range test, SPSS 20.0).

In conclusion, chloroplast-generated ROS play a main role in regulating NaCl-induced K^+ efflux in wheat leaf mesophyll. Both reducing NSCC sensitivity to ROS and alleviating ROS generation in chloroplast may be instrumental in improving mesophyll K^+ retention ability in wheat. However, the identity of specific NSCC channels has to be revealed in order to control NaCl-induced K^+ efflux. Additionally, pyramiding the ROS regulated mesophyll K^+ retention trait with other important traits (e.g. Na^+ exclusion) might be a promising way to improve salinity tolerance in wheat. Furthermore, as ROS play a dual role in plant salt tolerance, understanding the network between K^+ transport and the dynamic processes of the generation and scavenging of different ROS species in plant cell under salt stress would benefit the deciphering of the complexity of plant salt tolerance mechanisms as well as promote the program of breeding salt tolerant crop varieties.

Reference

Ache P, Becker D, Deeken R, Dreyer I, Weber H, Fromm J, Hedrich R (2001) VFK1, a *Vicia faba* K^+ channel involved in phloem unloading. *Plant J* **27**:571–580

Adem GD, Roy SJ, Plett DC, Zhou M, Bowman JP, Shabala S (2015) Expressing *AtNHX1* in barley (*Hordeum vulgare* L.) does not improve plant performance under saline conditions. *Plant Growth Regul* 77: 289−297

Adem GD, Roy SJ, Zhou M, Bowman JP, Shabala S (2014) Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. *BMC Plant Biol* **14**: 113

Ahmad P, Jaleel CA, Salem MA, Nabi G, Sharma S (2010) Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Crit Rev Biotechnol **30**: 161–175

Ahmad I, Maathuis FJM (2014) Cellular and tissue distribution of potassium: Physiological relevance, mechanisms and regulation. *J Plant Physiol* **171**: 708−714

Agarie A, Shimoda T, Shimizu Y, Baumann K, Sunagawa H, Kondo A, Ueno O, Nakahara T, Nose A, Cushman JC (2007) Salt tolerance, salt accumulation, and ionic homeostasis in an epidermal bladder-cell-less mutant of the common ice plant *Mesembryanthemum crystallinum*. *J Exp Bot* **58**: 1957−1967

Agrawal PB, Nierstrasz VA, Klug-Santner BG, Gübitz GM, Lenting HBM, Warmoeskerken MMCG (2007) Wax removal for accelerated cotton scouring with alkaline pectinase. *Biotech J* **2**: 306–315

Al-Karaki GN (2000) Growth, water use efficiency, and sodium and potassium acquisition by tomato cultivars grown under salt stress. *J Plant Nurt* **23**: 1−8

Allakhverdiev AI, Murata N (2008) Salt stress inhibits photosystems II and I in cyanobacteria. *Photosynth Res* **98**: 529−539

Allakhverdiev AI, Sakamoto A, Nishiyama Y, Inaba M, Murata N (2000) Ionic and osmotic effects of NaCl-induced inactivation of photosystem I and II in *Synechococcus* sp. *Plant Physiol* **123**: 1047−1056

Almeida P, Freon R, de Boer GJ, de Boer AH (2014) Role of Na^+, K^+, Cl^-, Pronline and sucrose concentrations in determining salinity tolerance and their correlation with the expression of multiple genes in tomato. *AOB Plants* **6**: plu039

Anil VS, Krishnamurthy H, Mathew MK (2007) Limiting cytosolic Na^+ confers salt tolerance to rice cells in culture: a two-photon microscopy study of SBFI-loaded cells. *Physiol Plant* **129**: 607–621

Anschütz U, Becker D, Shabala S (2014) Going beyond nutrition: regulation of potassium homoeostasis as a common denominator of plant adaptive responses to environment. *J Plant Physiol* **171**: 670–687

Apel K, Hirt H (2004) Reacitve oxygen species: metabolism. Oxidative stress, and signal transduction. *Annu Rev Plant Biol* **55**: 373−399

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science 258: 1256−1258.

Apse MP, Blumwald E (2007) Na^+ transport in plants. *FEBS Lett* **581**: 2247−2254

Asch F, Dingkuhn M, Dörffling K, Miezan K (2000) Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. *Euphytica* **113**: 109−118

Ashley MK, Grant M, Grabov A (2006) Plant responses to potassium deficiencies: a role for potassium transport proteins. *J Exp Bot* **57**: 425−436

Ashraf M, Harris PJC (2004) Potential biochemical indicators of salinity tolerance in plants. *Plant Sci* **166**: 3−16

Ashraf M, Khanum A (1997) Relationship between ion accumulation and grworh in two spring wheat lines differing in salt tolerance at different growth stages. *J Agron Crop Sci* **178**: 39−51

Baik BK, Ullrich SE (2008) Barley for food: Characteristics, improvement, and renewed interest. *J Cereal Sci* **48**: 233−242

Ball MC, Chow WS, Anderson JM (1987) Salinity-induced potassium deficiency causes loss of functional photosystem II in leaves of the grey mangrove, *Avicennia marina*, through depletion of the atrazine-binding polypeptide. *Aust J Plant Physiol* **14**: 351−361

Baluška F, Mancuso S (2013) Root apex transition zone as oscillatory zone. *Front Plant Sci* **4**: 1–15

Baluška F, Mancuso S, Volkmann D, Barlow P (2010) Root apex transition zone: a signalling-response nexus in the root. *Trends Plant Sci* **15**: 402–408

Banjara M, Zhu L, Shen G, Payton P, Zhang H (2012) Expression of an *Arabidopsis* sodium/proton antiporter gene (*AtNHX1*) in peanut to improve salt tolerance. *Plant Biotechnol Rep* **6**: 59−67

Barhoumi Z, Djebali W, Smaoui A, Chaïbi W, Abdelly C (2007) Contribution of NaCl excretion to salt resistance of *Aeluropus littoralis* (Willd) Parl. *J Plant Pysiol* **164**: 842−850

Barragán V, Leidi EO, Andrés Z, Rubio L, De Luca A, Fernández JA, Cubero B, Pardo JM (2012) Ion exchangers NHX1 and NHX2 meidate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in *Arabidopsis*. *Plant Cell* **24**: 1127–1142

Bassil E, Ohto M, Esumi T, Tajima H, Zhu Z, Cagnac O, Belmonte M, Peleg Z, Yamaguchi T, Blumwald E (2011) The *Arabidopsis* intracellular Na^+/H^+ antiporters NHX5 and NHX6 are endosome associated and necessary for plant growth and development. *Plant Cell* **23**: 224−239

Bednarz CW, Oosterhuis DM (1999) Physiological changes associated with potassium deficiency in cotton. *J Plant Nurt* **22**: 303−313

Benito B, Rodríguez-Navarro A (2003) Molecular cloning and characterization of a sodium-pump ATPase of the moss *Physcomitrella patens*. *Plant J* **36**: 382−389

Berthomieu P, Conéjéro G, Nublat A, Brackenbury WJ, Lambert C, Savio C, Uozumi N, Oiki S, Yamada K, Cellier F, Gosti F, Simonneau T, Essah PA, Tester M, Véry AA, Sentenac H, Casse F (2003) Functional analysis of *AtHKT1* in *Arabidopsis* shows that Na^+ recirculation by the phloem is crucial for salt tolerance. *EMBO J* **22**: 2004−2014

Bewick TA, Shilling DG, Querns R (1993) Evaluation of epicuticular wax removal from whole leaves with chloroform. *Weed Tech* **7**: 706–716

Binzel ML, Hess FD, Bressan RA, Hasegawa PM (1988) Intracellular compartmentation of ions in salt adapted tobacco cells. *Plant Physiol* **86**: 607−614

Blumwald E (2000) Sodium transport and salt tolerance in plants. *Curr Opin Cell Biol* **12**: 431−434

Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. *Biochim Biophys Acta* **1465**: 140−151

Bonales-Alatorre E, Pottosin I, Shabala L, Chen ZH, Zeng F, Jacobsen SE, Shabala S (2013a) Differential activity of plasma and vacuolar membrane transporters contributes to genotypic differences in salinity tolerance in a halophyte species, *Chenopodium quinoa*. *Int J Mol Sci* **14**: 9267–9285

Bonales-Alatorre E, Shabala S, Chen ZH, Pottosin I (2013b) Reduced tonoplast fast-activating and slow-activating channel activity is essential for conferring salinity tolerance in a facultative halophyte, quinoa. *Plant Physiol* **162**: 940–952

Boscari A, Clément M, Volkov V, Colldack D, Hybiak J, Miller AJ, Amtmann A, Fricke W (2009) Potassium channels in barley: cloning, functional characterization and expression analyses in relation to leaf growth and development. *Plant Cell Environ* **32**: 1761−1777

Bose J, Rodrigo-Moreno A, Shabala S (2014a) ROS homeostasis in halophytes in the context of salinity stress tolerance. *J Exp Bot* **65**: 1241−1257

Bose J, Shabala L, Pottosin I, Zeng F, Velarde-Buendía A, Massart A, Poschenrieder C, Hariadi Y, Shabala S (2014b) Kinetics of xylem loading, membrane potential maintenance, and sensitivity of K^+-permeable channels to reactive oxygen species: physiological traits that differentiate salinity tolerance between pea and barley. *Plant Cell Environ* **37**: 589–600

Britto DT, Kronzucker HJ (2008) Cellular mechanisms of potassium transport in plants. *Physiol Plant* **133**: 637–650

Byrt CS, Platten, JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R (2007) HKT1;5-like cation transporters linked to Na^+ exclusion loci in wheat, *Nax2* and *Kna1*. *Plant Physiol* **143**: 1918–1928

Byrt CS, XU B, Krishnan M, Lightfoot DJ, Athman A, Jacobs AK, Watson-Haigh NS, Munns R, Tester M, Gilliham M (2014) The Na^+ transporter, *TaHKT1;5-D*, limits shoot Na^+ exclusion in bread wheat. *Plant J* **80**: 516–526

Brini F, Hanin M, Mezghani I, Berkowitz GA, Masmoudi K (2007) Overexpression of wheat Na^+/H^+ antiporter *TNHX1* and H^+-pyrophosphatase *TVP1* improve salt- and drought-stress tolerance in *Arabidopsis thaliana* plants. *J Exp Bot* **58**: 301–308

Cakmak I (2005) The role of potassium in alleviating detrimental effects of abiotic stresses in plants. *J Plant Nutr Soil Sci* **168**: 521−530

Carden DE, Walker DJ, Flowers TJ, Miller AJ (2003) Single-cell measurements of the contributions of cytosolic Na^+ and K^+ to salt tolerance. *Plant Physiol* **131**: 676−683

Cavalcanti FR, Lima JPMS, Ferreira-Silva SL, Viégas RA, Silveria JAG (2007) Roots and leaves display contrasting oxidative response during salt stress and recovery in cowpea. *J Plant Physiol* **164**:591–600

Chen H, An R, Tang JH, Cui XH, Hao FS, Chen J, Wang XC (2007a) Over-expression of a vacuolar Na^+/H^+ antiporter gene improves salt tolerance in an upland rice. *Mol Breeding* **19**: 215−225

Chen J, Xiao Q, Wu F, Dong X, He J, Pei Z, Zheng H (2010) Nitric oxide enhances salt secretion and Na^+ sequestration in a mangrove plant, *Avicennia marina*, through

increasing the expression of H^+-ATPase and Na^+/H^+ antiporter under high salinity. *Tree Physiol* **30**: 1570–1585

Chen Z, Cuin TA, Zhou M, Twomey A, Naidu BP, Shabala S (2007b) Compatible solute accumulation and stress-mitigating effects in barley genotype contrasting in their genotype. *J Exp Bot* **58**: 4245–4255

Chen Z, Pottosin II, Cuin TA, Fuglsang AT, Tester M, Jha D, Zepeda-Jazo I, Zhou M, Palmgren MG, Newman IA, Shabala S (2007c) Root plasma membrane transporters controlling K^+/Na^+ homeostasis in salt-stressed barley. *Plant Physiol* **145**: 1714−1725

Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S (2005) Screening plants for salt tolerance by measuring K^+ flux: a case study for barley. *Plant Cell Environ* **28**:1230−1246

Chen Z, Zhou M, Newman IA, Mendham NJ, Zhang GP, Shabala S (2007d) Potassium and sodium relations in salinised barley tissues as a basis of differential salt tolerance. *Funct Plant Biol* **34**: 150–162

Chérel I, Lefoulon C, Boeglin M, Sentenac H (2014) Molecular mechanisms involved in plant adaption to low K^+ availability. *J Exp Bot* **65**: 833−848

Choi WG, Toyota M, Kim SH, Hilleary R, Gilroy S (2014) Slat stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. *Proc Nat Acad Sci* **111**: 6497−6502

Chow WS, Ball MC, Anderson JM (1990) Growth and photosynthetic response of spinach to salinity: implications of K^+ nutrition for salt tolerance. *Aust J Plant Physiol* **17**: 563−578

Christmann A, Grill E, Huang J (2013) Hydraulic signals in long-distance signaling. *Curr Opin Plant Sci* **16**: 293−300

Claes B, Dekeyser R, Villarroel R, Van den Bulcke M, Bauw G, Van Montagu M, Caplan A (1990) Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. *Plant Cell* **2**: 19–27

Colmenero-Flores JM, Martínez G, Gamba G, Vázquez N, Iglesias DJ, Brumós J, Talón M (2007) Identification and functional characterization of cation-chloride cotransporters in in plants. *Plant J* **50**: 278–292

Colmer TT, Flowers TJ, Munns R (2006) Use of wild relatives to improve salt tolerance in wheat. *J Exp Bot* **57**: 1059−1078

Colmer TD, Munns R, Flowers TJ (2005) Improving salt tolerance of wheat and barley: future prospects. *Aust J Exp Agric* **45**: 1425−1443

Conn S, Gilliham M (2010) Comparative physiology of elemental distributions in plants. *Ann Bot* **105**: 1081−1102

Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Poel BV, Pieters L, Vlietinck AJ, Berghe DV (1998) Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod* **61**: 71−76

Cramer GR, Läuchli A, Polito VS (1985) Displacement of Ca^{2+} by Na^{+} from the plasmalemma of root cells. A primary response to salt stress? *Plant Physiol* **79**: 207–211

Cuin TA, Betts SA, Chalmandrier R, Shabala S (2008) A root's ability to retain K^{+} correlates with salt tolerance in wheat. *J Exp Bot* **59**: 2697−2706

Cuin TA, Bose J, Stefano G, Jha D, Tester M, Mancuso S, Shabala S (2011) Assessing the role of root plasma membrane and tonoplast Na^{+}/H^{+} exchangers in salinity tolerance in wheat: in *planta* quantification methods. *Plant Cell Environ* **34**: 947−961

Cuin TA, Miller AJ, Laurie SA, Leigh RA (2003) Potassium activities in cell compartmetns of salt-grown barley leaves. *J Exp Bot* **54**: 657−611

Cuin TA, Parsons D, Shabala S (2010) Wheat cultivars can be screened for NaCl salinity tolerance by measuring leaf chlorophyll content and shoot sap potassium. *Funct Plant Biol* **37**: 656−664

Cuin TA, Tian Y, Betts SA, Chalmandrier R, Shabala S (2009) Ionic relations and osmotic adjustment in durum and bread wheat under saline conditions. *Funct Plant Biol* **36**: 1110–1119

Cuin TA, Zhou M, Parsons D, Shabala S (2012) Genetic behaviour of physiological traits conferring K^{+}/Na^{+} homeostasis in wheat. *Plant Biol* **14**: 438–446

Czempinski K, Frachisse JM, Maurel C, Barbier-Brygoo H, Muller-Roeber B (2002) Vacuolar membrane localization of the Arabidopsis 'two-pore' K^{+} channel KCO1. *Plant J* **29**: 809–820

Dagostino M, Lee CO (1982) Neutral carrier Na^{+}- and Ca^{2+}- selective microelectrodes for intracellular application. *Biophys J* **40**: 199–207

Dasgan HY, Aktas H, Abak K, Cakmak I (2002) Determination of screening techniques to salintiy tolerance in tomatoes and investigation of genotype responses. *Plant Sci* **163**: 695–703

Davies MJ, Poole RJ, Rea PA, Sanders D (1992) Potassium transport into plant vacuoles energized directly by a proton-pumping inorganic pyrophosphatase. *Proc Natl Acad Sci USA* **89**: 11701–11705

Davenport R (2002) Glutamate receptors in plants. *Ann Bot* **90**: 549−557

Davenport R, James RA, Zakrisson-Plogander A, Tester M, Munns R (2005) Control of Sodium transport in durum wheat. *Plant Physiol* **137**: 807−818

Davenport RJ, Muñoz-Mayor A, Jha D, Essah PA, Rus A, Tester M (2007) The Na^+ transporter AtHKT1;1 controls retrieval of Na^+ from the xylem in *Arabidopsis*. *Plant Cell Environ* **30**: 497−507

Davenport RJ, Reid RJ, Smith FA (1997) Sodium-calcium interactions in two wheat species differing in salinity tolerance. *Physiol Plant* **99**: 323−327

Dehdari A, Rezai A, Maibody SAM (2007) Genetic control of salt tolerance in wheat plants using generation means and variances analysis. *Water Soil Sci* **11**: 179–192

Degl'Innocenti E, Hafsi C, Guidi L, Navari-Izzo F (2009) The effect of salinity on photosynthetic activity in potassium-deficient barley species. *J Plant Physiol* **166**: 1968–1981

De Marchi U, Checchetto V, Zanetti M, Teardo E, Soccio M, Formentin E, Giacometti MG, Pastore D, Zoratti M, Szabò I (2010) ATP-sensitive cation-channel in wheat (*Triticum durum* Desf.): identification and characterization of a plant mitochondrial channel by patch-clamp. *Cell Physiol Biochem* **26**: 975–982

Demidchik V (2014) Mechanisms and physiological roles of K^+ efflux from root cells. *J Plant Physiol* **171**: 696–707

Demidchik V, Cuin TA, Svistunenko D, Smith SJ, Miller AJ, Shabala S, Sokolik A, Yurin V (2010) Arabidopsis root K^+-efflux conductance activated by hydroxyl radicals: singal-channel properties, genetic basis and involvement in strsss-induced cell death. *J Cell Sci* **123**: 1468–1479

Demidchik V, Davenport RJ, Tester M (2002) Nonselective cation channels in plants. *Annu Rev Plant Bio* **53**: 67–107

Demidchik V, Maathuis JM (2007) Physiological roles of nonselcetive cation channels in plant: from salt stress to signalling and development. *New Phytol* **175**: 387–404

Demidchik V, Shabala SN, Coutts KB, Tester MA, Davies JM (2003) Free oxygen radicals regulate plasma membrane Ca^{2+}-and K^+-premeable channels in plant root cells. *J Cell Sci* **116**: 81–88

Demidchik V, Shabala SN, Davies JM (2007) Spatial variation in H_2O_2 response of *Arabidopsis thaliana* root epidermal Ca^{2+} flux and plasma membrane Ca^{2+} channels. *Plant J* **49**: 377–386

Demidchik V, Straltsova D, Medvedev SS, Pozhvanov GA, Sokolik A, Yurin V (2014) Stress-induced electrolyte leakage: the role of K^+–premeable channels and involvement in programmed cell death and metabolic adjustment. *J Exp Bot* **65**: 1259–1270

Demidchik V, Tester M (2002) Sodium fluxes through nonselective cation channels in the plasma membrane of protoplasts from Arabidopsis roots. *Plant Physiol* **128**: 379–387

Dennison KL, Robertson WR, Lewis BD, Hirsch RE, Sussman MR, Spalding EP (2001) Functions of AKT1 and AKT2 potassium channels determined by studies of single and double mutants of Arabidopsis. *Plant Physiol* **127**: 1012–1019

Diatloff E, Forde BG, Roberts SK (2006) Expression and transport characterisation of the wheat low-affinity cation transporter (LCT1) in the methylotrophic yeast *Pichia pastoris*. *Biochem Biophy Res Commun* **344**: 807–813

Dietz KJ Schramm M, Lang B, Lanzl-Schramm A, Dürr C, Martinoia E (1992) Characterization of the epidermis from barley primary leaves. II: The role of the epidermis in ion compartmentation. *Planta* **187**: 431–437

Dinneny JR (2010) Analysis of the salt-stress response at cell-type resolution. *Plant Cell Environ* **33**: 54–3551

Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN (2008) Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. *Science* **320**: 942–945

Dodd AN, Kudla J, Snaders D (2010) The language of calcium signaling. *Annu Rev Plant Biol* **61**: 593−620

Doncheva S, Amenos M, Poschenrieder C, Barcelo J (2005) Root cell patterning: a primary target for aluminium toxicity in mazie. *J Exp Bot* **56**: 1213−1220

Dreyer I, Uozum N (2011) Potassium channels in plant cells. *FEBS J* **278**: 4293–4303

Dubcovsky J, María GS, Epstein E, Luo MC, Dvořák J (1996) Mapping of the K^+/Na^+ discrimiantion locus *Kna1* in wheat. *Theor Appl Genet* **92**: 448–454

Dvořák J, Gorham J (1992) Methodology of gene transfer by homoeologous recombination into *Triticum turgidum*: transfer of K^+/Na^+ discrimination from *Triticum aestivum*. *Genome* **35**: 639–646

Dvořák J, Noaman MM, Goyal S, Gorham J (1994) Enhancement of the salt tolerance of *Triticum turgidum* L. by the *Kna1* locus transferred from the *Triticum aestivum* L. chromosome 4D by homoeologous recombination. *Theor Appl Genet* **87**: 872–877

Eleuch L, Jilal A, Grando S, Ceccarelli S, von Korff Schmising M, Tsujimoto H, Hajer A, Daaloul A, Baum M (2008) Genetic diversity and association analysis for salinity tolerance, heading date and plant height of barley germplasm using simple sequence repeat markers. *J Integr Plant Biol* **50**:1004–1014

El-Hendawy SE, Hu Y, Yakout GM, Awad AM, Hafiz AE, Schmidhalter U (2005) Evaluating salt tolerance of wheat genotypes using multiple parameters. *Europ J Agron* **22**: 243–253

Elmore JM, Coaker G (2011) The role of the plasma membrane H^+-ATPase in Plant-microbe interactions. *Mol Plant* **4**:416–427

Eigenbrode SD, Espelie KE (1995) Effects of plant epicuticular lipids on insect herbivores. *Annu Rev Entomol* **40**: 171–194

Essa TA (2002) Effect of salinity stress on growth and nutrient composition of three soybean (*Glycine max* L. Merrill) cultivars. *J Agron Crop Sci* **188**: 86–93

Evans HJ, Sorger GJ (1966) Role of mineral elements with emphasis on the univalent cations. *Annu Rev Plant Physiol* **17**:47–76

Fan Y, Zhu M, Shabala S, Li CD, Johnson P, Zhou MX (2014) Antioxidant activity in salt-stressed barley leaves: evaluating time- and age-dependence and suitability for the use as a biochemical marker in breeding programs. *J Agron Crop Sci* **200**: 261–272

Fang Z, Mi F, Berkowitz GA (1995) Molecular and physiological analysis of a thylakoid K^+ channel protein. *Plant Physiol* **108**:1725–1734

FAO. (2013) FAO statistical yearbook (2013): world food and agriculture. 2013

Feki K, Quintero FJ, Pardo JM, Masmoudi K (2011) Regulation of durum wheat Na^+/H^+ exchanger TdSOS1 by phosphorylation. *Plant Mol Biol* **76**: 545–556

Flowers TJ (2004) Improving crop salt tolerance. *J Exp Bot* **55**: 307–319

Flowers TJ, Colmer TD (2008) Salinity tolerance in halophytes. *New Phytol* **179**: 945−963

Flowers TJ, Colmer TD, Munns R (2015) Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes. *Ann Bot* **115**: 419-431

Flowers TJ, Hajibagheri MA (2001) Salinity tolerance in *Hordeum vulgare*: ion concentrations in roots cells of cultivars differing in salt tolerance. *Plant Soil* **231**: 1–9

Flowers TJ, Galal HK, Bromham L (2010) Evolution of halophytes: multiple origins of salt tolerance in land plants. *Funct Plant Biol* **37**: 604–612

Flowers TJ, Yeo AR (1995) Breeding for salinity resistance in crop plants: where next? *Aust J Plant Physiol* **22**: 875–884

Fortmeier R, Schubert S (1995) Salt tolerance of maize (*Zea mays* L.): the role of sodium exclusion. *Plant Cell Environ* **18**: 1041−1047

Fricke W, Leigh RA, Tomos AD (1996) The intercellular distribution of vacuolar solutes in the epidermis and mesophyll of barley leaves changes in response to NaCl. *J Exp Bot* **47**: 1413−1426

Fricke W, Pritchard J, Leigh RA, Tomos AD (1994) Cells of the upper and lower epidermis of barley (*Hordeum vulgare* L.) leaves exhibit distinct patterns of vacuolar solutes. *Plant Physiol* **104**: 1201−1208

Fujibe T, Saji H, Arakawa K, Yabe N, Takeuchi Y, Yamamoto KT (2004) A methyl viologen-resistant mutant of Arabidopsis, which is allelic to ozone-sensitive *rcd1*, is tolerant to supplemental ultraviolet-B irradiation. *Plant Physiol* **134**: 275–285

Fukuda A, Chiba K, Maeda M, Nakamura A, Maeshima M, Tanaka Y (2004a) Effect of salt and osmotic stresses on the expression of genes for the vacuolar H^+-pyrophosphatase, H^+-ATPase subunint A, and Na^+/H^+ antiporter from barley. *J Exp Bot* **55**: 585–594

Fukuda A, Nakamura A, Tagiri A, Tanaka H, Miyao A, Hirochika H, Tanaka, Y (2004b) Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. *Plant Cell Physiol* **45**: 146–159

Galva C, Artigas P, Gatto C (2012) Nuclear Na^+/K^+ ATPase plays an active role in nucleoplasmic Ca^{2+} homeostasis. *J Cell Sci* **125**: 6137−6146

Garthwaite AJ, von Bothmer R, Colmer TD (2005) Salt tolerance in wild *Hordeum* species is associated with restricted entry of Na^+ and Cl^- into the shoots. *J Exp Bot* **56**: 2365–2378

Genc Y, McDonald GK, Tester M (2007) Reassessment of tissue Na^+ concentration as a criterion for salinity tolerance in bread wheat. *Plant Cell Environ* **30**: 1486–1498

Genc Y, Oldach K, Verbyla AP, Lott G, Hassan M, Tester M, Wallwork H, McDonald GK (2010) Sodium exclusion QTL associated with improved seedling growth in bread wheat under salinity stress. *Theor Appl Genet* **121**: 877−894

Gierth M, Mäser P (2007) Potassium transporters in plants – involvement in K^+ acquisition, redistribution and homeostasis. *FEBS Lett* **581**: 2348−2356

Gill SS, Tuteja N (2001) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 2010; 48: 909–930

Gilroy S, Suzuki N, Miller G, Choi WG, Toyota M, Devireddy AR, Mittler R (2014) A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. *Trend Plant Sci* **19**: 623−630

Glenn EP, Brown JJ, Blumwald E (1999) Slat tolerance and crop potential of halophytes. *Crit Rev Plant Sci* **18**: 227–255

Gorham J (1990) Salt tolerance in the triticeae: K/Na discrimination in synthetic hexaploid wheat. *J Exp Bot* **41**: 623–627

Gorham J, Hardy C, Wyn Jones RG, Joppa LR, Law CN (1987) Chromosomal location of a K/Na discrimination character in the D genome of wheat. *Theor Appl Genet* **74**: 584–588

Gouiaa S, Khoudi H, Leidi EO, Pardo JM, Masmoudi K (2012) Expression of wheat Na^+/H^+ antiporter *TNHXS1* and H^+ - pyrophosphatase *TVP1* genes in tobacco from a bicistronic transcriptional unit improves salt tolerance. *Plant Mol Biol* **79**: 137−155

Grattan SR, Grieve CM (1922) Mineral element acquisition and growth response of plants grown in saline environments. *Agr Ecosyst Environ* **38**: 275−300

Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophytes. *Annu Rev Plant Physiol* **31**:149–190

Gregorio GB, Senadhira D, Mendoza RD, Manigbas NL, Roxas JP, Guerta CQ (2002) Progress in breeding for salinity tolerance and associated abiotic stresses in rice. *Field Crop Res* **76**: 91−101

Gupta PK, Varshney RK, Sharma PC, Ramesh B (1999) Molecular markers and their applications in wheat breeding. *Plant Breeding* **118**: 369–390

Hacham Y, Holland N, Butterfield C, Ubeda-Tomas S, Bennett MJ, Chory J, Savaldi-Goldstein S (2011) Brassinosteroid perception in the epidermis controls root meristem size. *Development* **138**: 839–848

Hajibagheri MA, Flowers TJ, Collins JC, Yeo AR (1988) A comparison of the methods of X-ray microanalysis, compartmental analysis and longitudinal ion profiles to estimate cytoplasmic ion concentration in two maize varieties. *J Exp Bot* **39**: 279−290

Hajibagheri MA, Harvey DMR, Flowers TJ (1984) Photosynthetic oxygen evolution in relation to ion contents in the chloroplasts of *Suaeda maritima*. *Plant Sci* **34**: 353–62

Halliwell B, Cross CE (1994) Oxygen-derived specoes: their relation to human disease and environmental stress. *Environ Health Perspect* **102**: 5–12

Halperin SJ, Lynch JP (2003) Effects of salinity on cytosolic Na^+ and K^+ in root hairs of *Arabidopsis thaliana*: in vivo measurements using the fluorescent dyes SBFI and PBFI. *J Exp Bot* **54**: 2035–2043

Halušková L, Valentovičová K, Huttová J, Mistrík I, Tamás L (2009) Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. *Plant Physiol Biochem* **47**: 1069–1074

Hamilton ES, Schlegel AM, Haswell ES (2015) United diversity: mechanosensitive ion channels in plants. *Annu Rev Plant Biol* **66**: 113–137

Haro R, Bañuelos MA, Quintero FJ, Rubio F, Rodríguez-Navarro A (1993) Genetic basis of sodium exclusion and sodium tolerance in yeast. A model for plants. *Physiol Plant* **89**: 868–874

Harvey DMR, Flowers TJ (1978) Determination of the sodium, potassium and chloride ion concentrations in the chloroplasts of the halophyte *Suaeda maritima* by non-aqueous cell fractionation. *Protoplasma* **97**: 337–49

Harvey DMR, Hall JL, Flowers TJ, Kent B (1981) Quantitative ion localization within *Suaeda maritima* leaf mesophyll cells. *Planta* **151**: 555–60

Hauser F, Horie T (2010) A conserved salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+/Na^+ ratio in leaves during salinity stress. *Plant Cell Environ* **33**: 552−565

He C, Pasapula SV, Luo S, Venkataramani S, Qiu X, Kuppu S, Kornyeyev D, Holaday AS, Auld A, Blumwald E, Zhang H (2007) Ectopic expression of *AtNHX1* in cotton (*Gossypium hirsutum* L.) increases proline content and enhances photosynthesis under salt stress conditions. *J Cotton Sci* **11**: 266–274

He C, Yang A, Zhang W, Gao Q, Zhang J (2010) Improved salt tolerance of transgenic wheat by introducing *betA* gene for glycine betaine synthesis. *Plant Cell Tiss Org* **101**: 65–78

Hedrich R, Schroeder JI (1989) The physiology of ion channels and electrogenic pumps in higher plants. *Annu Rev Plant Physiol* **40**:539–569

Hepler PK (2005) Calcium: a central regulator of plant growth and development. *Plant Cell* **17**: 2142–2155

Hernández I, Alegre L, Munne-Bosch S (2006) Enhanced oxidation of falvan-3-ols and proanthocyanidin accumulation in water-stressed tea plants. *Phytochem* **67**: 1120–1126

Hernández I, Alegre L, van Breusegem F, Munne-Bosch S (2009) How relevant are flavonoids as antioxidants in plants. *Trends Plant Sci* **14**: 125–132

Hernández JA, Corpas FJ, Gómez M, del Río LA, Sevilla F (1993) Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. *Physiol Plant* **89**: 103−110

Hernández JA, Olmos E, Corpas FJ, Sevilla F, del Río LA (1995) Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Sci* **105**: 151–167

Horie T, Schroeder JI (2004) Sodium transporters in plants. Diverse genes and physiological functions. *Plant Physiol* **136**: 2457–2462

Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in *Arabidopsis* and monocot crop plants. *Trends Plant Sci* **14**: 660–668

Hosy E, Vavasseur A, Mouline K, Dreyer I, Gaymard F, Porée F, Boucherez J, Lebaudy A, Bouchez D, Véry A, Simonneau T, Thibaud J, Sentenac H (2003) The *Arabidopsis* outward K^+ channel *GORK* is involved in regulation of stomatal movements and plant transpiration. *Proc Nat Acad Sci* **100**:5549–5554

Huang CX, van Steveninck RFM (1988) Effect of moderate salinity on patterns of potassium, sodium and chloride accumulation in cells near the root tip of barley: Role of differentiating metaxylem vessels. *Physiol Plant* **73**: 525−533

Huang CX, van Steveninck RFM (1989) Maintenance of low Cl^- concentrations in mesophyll cells of leaf blades of barley seedlings exposed to salt stress. *Plant Physiol* **90**: 1440-1443

Huang CX, van Steveninck RFM (1990) Salinity induced structural changes in meristematic cells of barley roots. *New Phytol* **115**: 17−22

Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Denni ES, Munns R (2006) A sodium transporter (HKT7) is a candidate for *Nax1*, a gene for salt tolerance in durum wheat. *Plant Physiol* **143**: 1718–1727

Huang S, Spielmeyer W, Lagudah ES, Munns R (2008) Comparative mapping of HKT genes in wheat, barley, and rice, key determinants of Na^+ transport, and salt tolerance. *J Exp Bot* **59** (4): 927–937

Huang X, Zhang Y, Jiao B, Chen G, Huang S, Guo F, Shen Y, Huang Z, Zhao B (2012) Overexpression of the wheat salt tolerance-related gene *TaSC* enhances salt tolerance in *Arabidopsis*. *J Exp Bot* **63**: 5463–5473

Hughes FM and Cidlowski JA (1999) Potassium is a critical regulator of apoptotic enzymes *in vitro* and *in vivo*. *Adv Enzyme Regul* **39**: 157–171

Huh GH, Damsz B, Matsumoto TK, Reddy MP, Rus AM, Ibeas JI, Narasimhan ML, Bressan RA, Hasegawa (2002) Salt causes ion disequilibrium-induced programeed cell death in yeast and plants. *Plant J* **29**: 649–659

Husain S, Munns R, Condon AGT (2003) Effect of sodium exclusion trait on chlorophyll retention and growth of durum wheat in saline soil. *Aust J Agric Res* **54**: 589−597

Husain S, von Caemmerer S, Munns R (2004) Control of salt transport from roots to shoots of wheat in saline soil. *Func Plant Biol* **31**: 1115−1126

Ioio RD, Linhares FS, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P, Sabatini S (2007) Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. *Curr Biol* **17**: 678−682

Ioio RD, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P, Sabatini S (2008) A genetic framework for the control of cell division and differentiation in the root meristem. *Science* **17**: 678−682

Iyer-Pascuzzi AS, Jackson T, Cui H, Petricka JJ, Busch W, Tsukagoshi H, Benfey PN (2011) Cell identity regulators link development and stress responses in the *Arabidopsis* root. *Dev Cell* **21**: 770−782

Jain M, Nijhawan A, Arora R, Agarwal P, Ray S, Sharma P, Kapoor S, Tyagi AK, Khurana JP (2007) F-Box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. *Plant Physiol* **143**: 1467−1483

James RA, Blake C, Byrt CS, Munns R (2011) Major genes for Na^+ exclusion, Nax1 and Nax2 (wheat HKT1;4 and HKT1;5), decrease Na^+ accumulation in bread wheat leaves under saline and waterlogged conditions. *J Exp Bot* **62**: 2939−2947

James RA, Davenport RJ, Munns R (2006a) Physiological characterization of two genes for Na^+ exclusion in durum wheat, *Nax*1 and *Nax*2. *Plant Physiol* **142**: 1537–1547

James RA, Munns R, von Caemmerer S, Trejo C, Miller C, Condon TA (2006b) Photosynthetic capacity is related to the cellular and subcellular partitioning of Na^+, K^+ and Cl^- in salt-affected barley and durum wheat. *Plant Cell Environ* **29**: 2185–2197

James RA, Rivelli AR, Munns R, von Caemmerer S (2002) Factors affecting CO_2 assimilation, leaf injury and growth in salt-stressed durum wheat. *Funct Plant Biol* **29**: 1393–1403

James RA, Blake C, Byrt CS, Munns R (2011) Major genes for Na^+ exclusion, *Nax1* and *Nax2* (wheat *HKT1;4* and *HKT1;5*), decrease Na^+ accumulation in bread wheat leaves under saline and waterlogged conditions. *J Exp Bot* **62**: 2939–2947

Jamil A, Riaz S, Ashraf M, Foolad MR (2011) Gene expression profiling of plants under salt stress. *Crit Rev Plant Sci* **30**: 435–458

Jayakannan M, Babourina O, Rengel Z (2011) Improved measurements of Na^+ fluxes in plants using calixarene-based microelectrodes. *J Plant Physiol* **168**: 1045–1051

Jeschke WD, Aslam Z, Greenway H (1986) Effects of NaCl on ion relations and carbohydrate status of roots and on osmotic regulation of roots and shoots of *Atriplex amnicola*. *Plant Cell Environ* **9**: 559–569

Jeschke WD, Pate JS (1991) Cation and chloride partitioning through xylem and phloem within the whole plant of *Ricinus communis* L. under conditions of salt stress. *J Exp Bot* **42**: 1105-1116

Jeschke WD, Stelter W (1976) Measurements of longitudinal ion profiles in single roots of *Hordeum* and *Atriplex* by use of flameless atomic absorption spectroscopy. *Planta* **128**: 107–112

Jha D, Shirley N, Tester M, Roy SJ (2010) Variation in salinity tolerance and shoot sodium accumulation in Arabidopsis ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport. *Plant Cell Environ* **33**: 793–804

Jiang X, Leidi EO, Pardo JM (2010) How do vacuolar NHX exchangers function in plant salt tolerance? *Plant Signal Behav* **5**: 792–795

Jin SH, Huang JQ, Li XQ, Zheng BS, Wu JS, Wang ZJ, Liu GH, Chen M (2011) Effects of potassium supply on limitations of photosynthesis by mesophyll conductance in *Carya cathayensis*. *Tree Physiol* **31**: 1142−1151

Johnson MK, Loo G (2000) Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutat Res* **459**: 211–218

Jung JY, Mo HC, Yang KH, Jeong YJ, Yoo HG, Choi NK, Oh WM, Oh HK, Kim SH, Lee JH, Kim HJ, Kim WJ (2007) Inhibition by epigallocatechin gallate of CoCl2-induced apoptosis in rat PC12 cells. *Life Sci* **80**: 1355–1363

Karley AJ, Leigh RA, Sanders D (2000) Differential ion accumulation and ion fluxes in the mesophyll and epidermis of barley. *Plant Physiol* **122**: 835−844

Katschnig D, Bliek T, Rozema J, Schat H (2015) Constitutive high-level *SOS1* expression and absence of *HKT1;1* expression in the salt accumulating halophyte *Salicornia dolichostachya*. *Plant Sci* **234**: 144−154

Katsuhara M, Kawasaki T (1996) Salt stress induced nuclear and DNA degradation in meristematic cells of barley roots. *Plant Cell Physiol* **37**: 169–173

Kerkeb L, Donaire JP, Rodríguez-Rosales MP (2001) Plasma membrane H+-ATPase activity is involved in adaption of tomato calli to NaCl. *Physiol Plant* **111**: 483–490

Kiegle E, Moore CA, Haseloff J, Tester MA, Knight MR (2000) Cell-type-specific calcium responses to drought, salt and cold in the *Arabidopsis* root. *Plant J* **23**: 267−278

Kim S, Kang JY, Cho DI, Park JH, Kim SY (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. *Plant J* **40**: 75−87

Kingsbury RW, Epstein E, Peracy RW (1984) Physiological responses to salinity in selected lines of wheat. *Plant Physiol Bioch* **74**: 417–423

Kinraide TB (1999) Interactions among Ca^{2+}, Na^{+} and K^{+} in salinity toxicity: quantitative resolution of multiple toxic and ameliorative effects. *J Exp Bot* **50**: 1495–1505

Kleyman TR, Gragoe EJ (1988) Amiloride and its analogs as tools in the study of ion transport. *J Membrane Biol* **105**: 1−21

Kobayashi H, Masaoka Y, Takahashi Y, Ide Y, Sato S (2007) Ability of salt glands in Rhodes grass (*Chloris gayana* Kunth) to secrete Na^{+} and K^{+}. *Soil Sci Plant Nutr* **53**: 764–771

Koch K, Barthlott W, Koch, S, Hommes A, Wandelt K, Mamdouh W, De-Feyter S, Broekmann P (2006) Structural analysis of wheat wax (*Triticum aestivum, c.v.* 'Naturastar' L.): from the molecular level to three dimensional crystals. *Planta* **223**: 258–270

Kong X, Luo Z, Dong H, Eneji AE, Li W (2012) Effects of non-uniform root zone salinity on water use, Na^{+} recirculation, and Na^{+} and H^{+} flux in cotton. *J Exp Bot* **63**: 2105–2116

Kong XQ, Guo XH, Sun W, An J, Zhao YX, Zhang H (2011) Cloning and functional characterization of a cation-chloride cotransporter gene *OsCCC1*. *Plant Mol Biol* **75**: 567–578

Koyama H, Toda T, Hara T (2001a) Brief exposure to low-pH stress causes irreversible damage to the growing root in *Arabidopsis thaliana*: pectin-Ca interaction may play an important role in proton rhizotoxicity. *J Exp Bot* **52**: 361−388

Koyama ML, Levesley A, Koebner RMD, Flowers TJ, Yeo AR (2001b) Quantitative trait loci for component physiological traits determining salt tolerance in rice. *Plant Physiol* **125**: 406–422

Koyro HW, Stelzer R (1988) Ion concentrations in the cytoplasm and vacuoles of rhizodermis cells from NaCl treated *Sorghum*, *Spartina* and *Puccinellia* plants. *J Plant Physiol* **133**: 441–446

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *J Exp Bot* **63**: 1593–1608

Krebs M, Beyhl D, Gorlich E, Al-Rasheid KAS, Marten I, Stierhof YD, Hedrich R, Schumacher K (2010) Arabidopsis V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proc Natl Acad Sci* **107**: 3251−3256

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. *Plant Physiol* **130**: 2129−2141

Kronzucker HJ, Britto DT (2011) Sodium transport in plants: a critical review. *New Phytol* **189**: 54–81

Kronzucker HJ, Coskun D, Schulze LM, Wong JR, Britto DT (2013) Sodium as nutrient and toxicant. *Plant Soil* **369**: 1–23

Kudla J, Batistic O, Hashimoto K (2012) Calcium signals: the lead currency of plant information processing. *Plant Cell* **22**: 541−563

Kurusu T, Kuchitsu K, Nakano M, Nakayama Y, Iida H (2013) Plant mechanosensing and Ca^{2+} transport. *Trend Plant Sci* **18**: 227−233

Laoli C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. *Curr Opin Plant Biol* **7**: 323−328

Lauchli A, James RA, Huang CX, McCully M, Munns R (2008) Cell-specific localization of Na^+ in roots of durum wheat and possible control points for salt exclusion. *Plant Cell Environ* **31**: 1565−1574

Läuchli A, Wieneke J (1979) Studies on growth and distribution of Na^+, K^+ and Cl^- in soybean varieties differing in salt tolerance. *Z. Pflanzenernaehr. Bodebnkd.* **142**: 3−13

Lee R (2011) The outlook for population growth. *Science* **333**: 569–573

Lee XZ, Liang YR, Chen H, Lu JL, Liang HL, Huang FP, Mamati EG (2008) Alleviation of UV-B stress in Arabidopsis using tea catechins. *Afr J Biotechnol* **7**: 4111–4115

Leigh (2001) Potassium homeostasis and membrane transport. *J Plant Nutr Soil Sci* **164**: 193–198

Leigh RA, Storey R (1993) Intercellular compartmentation of ions in barley leaves in relation to potassium nutrition and salinity. *J Exp Bot* **44**: 755−762

Leigh RA, Tomos AD (1993) Ion distribution in cereal leaves: pathways and mechanisms. *Phil Trans R Soc Lond B* **341**: 75−86

Leigh RA, Wyn Jones RG (1984) A hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant cell. *New Phytol* **97**: 1–13

Li J, Pu L, Han M, Zhu M, Zhang R, Xiang Y (2014a) Soil salinization research in China: Advances and prospects. *J Geogr Sci* **24**: 943−960

Li R, Zhang J, Wu G, Wang H, Chen Y, Wei J (2012) HbCIPK2, a novel CBL-interacting protein kinase from halophyte *Hordeum brevisubulatum*, confers salt and osmotic stress tolerance. *Plant Cell Environ* **35**: 1582–1600

Liang Y (1999) Effects of silicon on enzyme activity and sodium, potassium and calcium concentration in barley under salt stress. *Plant Soil* **209**: 217–224

Liang Y, Mitchell DM, Harris JM (2007) Abscisic acid rescues the root meristem defects of the *Medicago truncatula latd* mutant. *Dev Biol* **304**: 297–307

Lindsay MP, Lagudah ES, Hare RA, Munns R (2004) A locus for sodium exclusion (Nax1), a trait for salt tolerance, mapped in durum wheat. *Funct Plant Biol* **31**: 1105–1114

Liphschitz N, Eshel ASLA, Waisel Y (1974) Salt glands on leaves of Rhodes grass (*Chloris gayana* Kth.). *Ann Bot* **38**: 459–462

Liu T, van Staden J, Cress WA (2000) salinity induced nuclear and DNA degradation in meristematic cells of soybean (*Glycine max* (L.)) roots. *Plant Growth Regul* **30**: 49−54

Livak KJ, Schmittgen, TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ methods. *Methods* **25**: 402−408

Löw R, Rockel B, Kirsch M, Ratajczak R, Hörtensteiner S, Martinoia E, Lüttge U, Rausch T (1996) Early salt stress effects on the differential expression of vacuolar H^+-ATPase genes in roots and leaves of *Mesembryanthemum crystallinum*. *Plant Physiol* **110**: 259−265

Luan S, Lan W, Lee SC (2009) Potassium nutrition, sodium toxicity, and calcium signalling: connections through the CBL-CIPK network. *Curr Opin Plant Biol* **12**: 339–346

Lunde C, Drew DP, Jacobs AK, Tester M (2007) Exclusion of Na^+ via sodium ATPase (PpENA1) ensures normal growth of *Physcomitrella patens* under moderate salt stress. *Plant Physiol* **144**: 1786−1796

Lv S, Nie L, Fan P, Wang X, Jiang D, Chen X, Li Y (2012) Sodium plays a more important role than potassium and chloride in growth of Salicornia europaea. Acta Physiol Plant 34: 503−513.

Ma JF, Shen R, Nagao S, Tanimoto E (2004) Aluminum targets elongating cells by reducing cell wall extensibility in wheat roots. *Plant Cell Physiol* **45**: 583−589

Maathuis FJM (2014) Sodium in plants: perception, signalling, and regulation of sodium fluxes. *J Exp Bot* **65**: 849−858

Maathuis FJM, Ahmad I, Patishtan J (2014) Regulation of Na^+ fluxes in plants. *Front Plant Sci* **5**: 467

Maathuis FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. *Ann Bot* **84**:123–133

Maathuis FJM, Ichida AM, Sanders D, Schroeder JI (1997) Roles of higher plant K^+ channels. *Plant Physiol* **114**: 1141–1149

Maathuis FJM, Sanders D (2001) Sodium uptake in Arabidopsis root is regulated by cyclic nucleotides. *Plant Physiol* **127**: 1617–1625

Maksimović JD, Zhang J, Zeng F, Živanović BD, Shabala L, Zhou M, Shabala S (2013) Linking oxidative and salinity stress tolerance in barley: can root antioxidant enzyme activity be used as a measure of stress tolerance? *Plant Soil* **365**: 141–155

Mäser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJM, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiol* **126**:1646–1667

Mano Y, Takeda K (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). *Euphytica* **94**:263–272

Mansour MMF, Salama KHA, Al-Mutawa MM (2003) Transport proteins and salt tolerance in plants. *Plant Sci* **164**: 891–900

Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, Kanesaki Y, Hagemann M, Murata N (2003) Identification of histidine kinases that act as sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. *Proc Natl Acad Sci USA* **100**: 9061–9066

Marschner H, Kirkby EA, Cakmak I (1996) Effect of mineral nutritional status on shoot-root partitioning of photoassimilates and cycling of mineral nutrients. *J Exp Bot* **47**:1255–1263

Mäser P, Eckelman B, Vaidyanathan R, Horie T, Fairbairn DJ, Kubo M, Yamagami M, Yamaguchi K, Nishimura M, Uozumi N, Robertson W, Sussman MR, Schroeder JI (2002a) Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in *Arabidopsis* by genetic disruption of the Na^+ transporter *AtHKT1*. *FEBS Lett* **531**: 157–161

Mäser P, Gierth M, Schroeder JI (2002b) Molecular mechanisms of potassium and sodium uptake in plants. *Plant Soil* **247**: 43–54

Mass EV, Poss JA (1989) Salt sensitivity odf wheat at various growth stages. *Irrig Sci* **10**: 29–40

Matsushita N, Matoh T (1991) Characterization of Na^+ exclusion mechanisms of salt-tolerant reed plants in comparison with salt-sensitive rice plants. *Physiol Plant* **83**: 170–176

Meier SD, Kovalchuk Y, Rose CR (2006) Properties of the new fluorescent Na^+ indicator CoroNa Green: Comparison with SBFI and confocal Na^+ imaging. *J Neurosci Meth* **155**: 251–259

Mian A, Oomen RJ, Isayenkov S, Sentenac H, Maathuis FJ, Very AA (2011) Over-expression of an Na^+-and K^+-permeable HKT transporter in barley improves salt tolerance. *Plant J* **68**: 468–479

Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, Dangl JL, Mittler R (2009) The plant NADPH oxidase RBOHD mediates rapid systemic signalling in response to diverse stimuli. *Sci Signal* **2**: ra45

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, Gollery M, Shulaev V, van Breusegem F (2011) ROS signaling: the new wave? *Trend Plant Sci* **16**: 300−309

Møller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M (2009) Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na^+ transport in Arabidopsis. *Plant Cell* **21**: 2163–2178

Møller IS, Tester M (2007) Salinity tolerance of Arabidopsis: a good model for cereals? *Trends Plant Sci* **12**: 534–540

Morsomme P, Boutry M (2000) The plant plasma membrane H^+ -ATPase: structure, function and regulation. *Biochim Biophys Acta* **1465**: 1–16

Munns R (2005) Salinity stress and its impact. In: Blum A, ed. *Plant Stress.*

Munns R, Hare RA, James RA, Rebetzke GJ (2000) Genetic variation for improving the salt tolerance of durum wheat. *Aust J Agric Res* **51**: 69–74

Munns R, Husain S, Rivelli AR, James RA, Condon AGT, Lindsay MP, Lagudah ES, Schachtman DP, Hare RA (2002) Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. *Plant Soil* **247**: 93–105

Munns R, James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant Soil* **253**: 201–218

Munns R, James RA, Läuchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. *J Exp Bot* **57**: 1025–1043

Munns R, James RA, Xu B, Athman A, Conn Sj, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M, Plett D, Gilliham M (2012) Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. *Nat Biotechnol* **30**: 360–366

Munns R, Rebetzke GJ, Husain S, James RA, Hare RA (2003) Genetic control of sodium exclusion in durum wheat. *Aust J Agric Res* **54**: 627–635

Munns R, Tester M (2008) Mechanism of salinity tolerance. *Annu Rev Plant Biol* **59**: 651–681

Muralitharan MS, Chandler S, Van Steveninck RFM (1992) Effects of NaCl and Na_2SO_4 on growth and solute composition of highbush blueberry (*Vaccinium corymbosum*). *Aust J Plant Physiol* **19**: 155–164

Nakagawa T, Yokozawa T (2002) Direct scavenging of nitric oxide and superoxide by green tea. *Food Chem Toxicol* **40**: 1745–1750

Nelson DE, Rammesmayer G, Bohnert HJ (1998) Regulation of Cell-specific inositol metabolism and transport in plant salinity tolerance. *Plant Cell* **10**: 753–764

Newman IA (2001) Ion transport in plants: measurement of fluxes using ion-selective microelectrodes to characterize transporter function. *Plant Cell Environ* **24**: 1–14

Newman IA, Kochian LV, Grusak MA, Lucas WJ (1987) Fluxes of H^+ and K^+ in corn roots: Characterization and stoichiometries using ion-selective microelectrodes. *Plant Physiol* **84**: 1177–1184

Nublat A, Desplans J, Casse F, Berthomieu P (2001) *sas1*, an Arabidopsis mutant over accumulating sodium in the shoot, shows deficiency in the control of the root radial transport of sodium. *Plant Cell* **13**: 125–137

Oh DH, Lee SY, Bressan RA, Yun DJ, Bohnert HJ (2010) Intracellular consequences of SOS1 deficiency during salt stress. *J Exp Bot* **61**: 1205–1213

Oh DH, Leidi E, Zhang Q, Hwang SM, Li Y, Quintero FJ, Jiang X, D'Urzo MP, Lee SY, Zhao Y, et al (2009) Loss of halophytism by interference with SOS1 expression. *Plant Physiol* **151**: 210–222

Olías R, Eljakaoui Z, Li J, de Morales PA, Marín-Manzano MC, Pardo JM, Belver A (2009a). The plasma membrane Na^+/H^+ antiporter SOS1 is essential for salt tolerance in tomato and affects the partitioning of Na^+ between plant organs. *Plant Cell Environ* **32**: 904–916

Olías R, Eljakaoui Z, Pardo JM, Belver A (2009b) the Na^+/H^+ exchanger SOS1 controls extrusion and distribution of Na^+ in tomato plants under salinity conditions. *Plant Signal Behav* **4**: 973–976

Osaki M, Shinano T, Tadano T (1993) Effect of nitrogen, phosphorus, or potassium deficiency on the accumulation of ribulose-1,5-bisphosphate carboxylase/oxygenase and chlorophyll in several field crops. *Soil Sci Plant Nutr* **39**: 417–425

Page MJ, Cera ED (2006) Role of Na^+ and K^+ in enzyme function. Physiol Rev **86**: 1049–1092

Palmgren MG (2001) Plant plasma membrane H^+–ATPase: powerhouses for nutrient uptake. *Annu Rev Plant Physiol Plant Mol Biol* **52**: 817–845

Pantoja O, Dainty J, Blumwald E (1989) Ion channels in vacuoles from halophytes and glycophytes. *FEBS Lett* **255**: 92–96

Pardo JM, Quintero FJ (2002) Plants and sodium ions: keeping company with the enemy. *Genome Biol* **3**: 1017.1–1017.4

Park M, Lee H, Lee JS, Byun MO, Kim BG (2009) *In planta* measurements of Na^+ using fluorescent dye CoroNa Green. *J Plant Biol* **52**: 298–302

Pedersen CN, Axelsen KB, Harper JF, Palmgren MG (2012) Evolution of plant P-type ATPase. *Front Plant Sci* **3**: 1–19

Perez-Lopez U, Robredo A, Lacuesta M, Munoz-Rueda A, Mena-Petite A (2010) Atmospheric CO_2 concentration influences the contributions of osmolyte accumulation and cell wall elasticity to salt tolerance in barley cultivars. *J Plant Physiol* **167**: 15–22

Peshev D, Vergauwen R, Moglia A, Hideg É, Van den Ende W (2013) Towards understanding vacuolar antioxidant mechanisms: a role for fructans? *J Exp Bot* **64**: 1025–1038

Peters J, Chin C (2007) Potassium loss is involved in tobacco cell death induced by palmitoleic acid ceramide. *Arch Biochem Biophys* **465**: 180–186

Pettigrew WT (2008) Potassium influences on yield and quality production for maize, wheat, soybean and cotton. *Physiol Plant* **133**: 670–681

Pilot G, Gaymard F, Mouline K, Chérel I, Sentenac H (2003) Regulated expression of *Arabidopsis* shaker K^+ channel genes involved in K^+ uptake and distribution in the plant. *Plant Mol Biol* **51**: 773–787

Pitman MG, Läuchli A, Stelzer R (1981) Ion distribution in roots of barley seedlings measured by electron probe X-ray microanalysis. *Plant Physiol* **68**: 673–679

Platten JD, Cotsaftis O, Berthomieu P, Bohbert H, Davenport RJ, Fairbairn DJ, Horie T, Leigh RA, Lin HX, Luan S, Mäser P, Pantoja O, Rodríguez-Navarro A, Schachtman DP, Schroeder JI, Sentenac H, Uozumi N, Véry AA, Zhu JK, Dennis ES, Tester M (2006) Nomenclature for HKT transporters, key determinants of plant salinity tolerance. *Trends Plant Sci* **11**: 372–374

Plett DC, Møller IS (2010) Na^+ transport in glycophytic plants: what we know and would like to know. *Plant Cell Environ* **33**: 612–626

Plett D, Safwat G, Gilliham M, Møller IS, Roy S, Shirley N, Jacobs A, Johnson A, Tester M (2010) Improved salinity tolerance of rice through cell type-spspecific expression of *AtHKT1;1*. *PLoS One* **5**: e12571

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci* **12**: 98–105

Pottosin I, Bonales-Alatorre E, Shabala S (2014a) Choline but not its derivative betaine blocks slow vacuolar channels in the halophyte *Chenopodium quinoa*: Implications for salinity stress responses. *FEBS Lett* **588**: 3918–3923

Pottosin I, Velarde-Buendía A, Bose J, Zepeda-Jado I, Shabala S, Dobrovinskaya O (2014b) Cross-talk between reactive oxygen species and polyamines in regulation of ion transport across the plasma membrane: implications for plant adaptive responses. *J Exp Bot* **65**: 1271–1283

Poustini K, Siosemardeh A (2004) Ion distribution in wheat cultivars in response to salinity stress. *Field Crop Res* **85**: 125–133

Qadir M, Quillerou E, Nangia V, Murtaza G, Singh M, Thomas RJ, Drechsel P, Noble AD (2014) Economics of salt-induced land degradation and restoration. *Nat Resour Forum* **38**: 282–295

Rahnama A, Poustini K, Tavakkol-Afshari R, Ahmadi A, Alizadeh H (2011) Growth properties and ion distribution in different tissues of bread wheat genotypes (*Triticum aestivum* L.) differing in salt tolerance. *J Agron Crop Sci* **197**: 21–30

Rajendran K, Tester M, Roy SJ (2009) Quantifying the three main components of salinity tolerance in cereals. *Plant Cell Environ* **32**: 237–249

Raven JA (1995) Scaling the seas. *Plant Cell Environ* **18**: 1090–1100

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat Genet* **37**: 1141–1146

Rengasamy P (2006) World salinization with emphasis in Australia. *J Exp Bot* **57**: 1017–1023

Regasamy P (2010) Soil processes affecting crop production in salt-affected soils. *Funct Plant Biol* **37**: 613–620

Rhee Y, Hlousek-Radojcic A, Ponsamuel J, Liu D, Post-Beittenmiller D (1998) Epicuticular wax accumulation and fatty acid elongation activities are induced during leaf development of leeks. *Plant Physiol* **116**: 901–911

Richardson KVA, Wetten AC, Caligari PDS (2001) Cell and nuclear degradation in root meristems following exposure of potatoes (*Solanum tuberosum* L.) to salinity. *Potato Res* **44**: 389–399

Rivandi J, Miyazaki J, Hrmova M, Pallotta M, Tester M, Collins NC (2010) A *SOS3* homologue maps to *HvNax4*, a barley locus controlling an environmentally sensitive Na^+ exclusion trait. *J Exp Bot* **62**: 1201–1216

Robinson SP, Downtown WJS (1984) Potassium, sodium, and chloride ion concentrations in leaves and isolated chloroplasts of the halophyte *Suaeda australis* R.Br. Aust J Plant Physiol **12**: 471–479

Robinson SP, Downtown WJS (1985) Potassium, sodium, and chloride content of isolated intact chloroplasts in relation to ionic compartmentation in leaves. *Arch Biochem Biophys* **228**: 197–206

Robinson SP, Downton WJS, Millhouse JA (1983) Photosynthesis and ion content of leaves and isolated chloroplasts of salt-stressed spinach. *Plant Physiol* **73**: 238–42

Rodríguez HG, Roberts JKM, Jordan WR, Drew MC (1997) Growth, water relations, and accumulation of organic and inorganic solutes in roots of maize seedlings during salt stress. *Plant Physiol* **113**: 881–893

Rodríguez-Navarro A, Rubio F (2006) High-affinity potassium and sodium transport systems in plants. *J Exp Bot* **57**: 1149–1160

Rodríguez-Rosales MP, Galvez FJ, Huertas R, Aranda MN, Baghour M, Cagnac O, Venema K (2009) Plant NHX cation/proton antiporters. *Plant Signal Behav* **4**: 265–276

Roy SJ, Huang W, Wang XJ, Evrard A, Schmöckel SM, Zafar ZU, Tester M (2013) A novel protein kinase involved in Na^+ exclusion revealed from positional cloning. *Plant Cell Environ* **36**: 553–568

Ruan CJ, da Silva JAT, Mopper S, Qin P, Lutts S (2010) Halophyte improvement for a salinized world. *Crit Rev Plant Sci* **29**: 329–359

Rubio F, Santa-María GE, Rodríguez-Navarro A (2000) Cloning of Arabidopsis and barley cDNAs encoding HAK potassium transporters in root and shoot cells. *Physiol Plant* **109**: 34–43

Rus A, Baxter I, Muthukumar B, Gustin J, Lahner B, Yakubova E, Salt DE (2006) Natural variants of At*HKT1* enhance Na^+ accumulation in two wild populations of *Arabidopsis*. *Plos Genet* **2**: e210

Rus A, Yokoi S, Sharkhuu A, Reddy M, Lee BH, Matsumoto TK, Koiwa H, Zhu JK, Bressan RA, Hasegawa PM (2001) AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots. *Proc Natl Acad Sci USA* **98**: 14150−14155

Saffari Y, Sadrzadeh SMH (2004) Green tea metabolite EGCG protects membranes against oxidative damage in vitro. *Life Sci* **74**: 1513–1518

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance in rice plants. *Plant J* **23**: 319–327

Saqib M, Akhtar J, Qureshi RH (2005a) Na^+ exclusion and salt resistance of wheat (*Triticum aestivum*) in saline-waterlogged conditions are improved by the development of adventitious nodal roots and cortical root aerenchyma. *Plant Sci* **169**: 125–130

Saqib M, Zorb C, Rengel Z, Schubert S (2005b) The expression of the endogenous vacuolar Na^+/H^+ antiporters in roots and shoots correlates positively with the salt resistance of wheat (*Triticum aestivm* L.). *Plant Sci* **169**: 959–965

Savouré A, Thorin D, Davey M, Hua XJ, Mauro A, van Montagu M, Inzé D, Verbruggen N (1999) NaCl and $CuSO_4$ treatments trigger distinct oxidative defence mechanisms in *Nicotiana plumbaginifolia* L. *Plant Cell Environ* **22**: 387–396

Schachtman DP, Kumar R, Schroeder JI, Marshi EL (1997) Molecular and functional characterization of a novel low-affinity cation transporter (LCT1) in higher plants. *Proc Natl Acad Sci UAS* **94**: 11079–11084

Schulze LM, Britto DT, Li M, Kronzucker HJ (2012) A pharmacological analysis of high affinity sodium transport in barley (*Hordeum vulgare* L.): a $^{24}Na^{+}/^{42}K^{+}$ study. *J Exp Bot* **63**: 2479–2489

Serrano A, Pérez-Castiñeira JR, Baltscheffsky MB, Baltscheffsky H (2007) H^{+}-PPases: Yesterday, Today and Tomorrow. *IUBMB Life* **59**: 76–83

Shabala S (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean mesophyll. *Plant Cell Environ* **23**: 825–837

Shabala S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. *Ann Bot* **92**: 627–634

Shabala S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Ann Bot* **112**: 1209-1221

Shabala S, Bose J, Hedrich R (2014) Slat bladders: do they matter? *Trend Plant Sci* **19**: 687–691

Shabala S, Cuin TA, Pottosin I (2007a) Polyamines prevent NaCl-induced K^{+}efflux from pea mesophyll by blocking non-selective cation channels. *FEBS Lett* **581**: 1993–1999

Shabala S, Cuin TA, Prismall L, Nemchinov, LG (2007b) Expression of animal CED -9 anti-apoptotic gene in tobacco modifies plasma membrane ion fluxes in response to salinity and oxidative stress. *Planta* **227**: 189–197

Shabala L, Cuin TA, Newman IA, Shabala S (2005) Salinity-induced ion flux patterns from the excised roots of *Arabidopsis sos* mutants. *Planta* **222**: 1041–1050

Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. *Physiol Plantarum* **133**: 651–669

Shabala S, Cuin TA, Shabala L, Newman I (2012) Quantifying kinetics of net ion fluxes from plant tissues by non-invasive microelectrode measuring MIFE technique. *Methods Mol Biol* **913**: 119−134

Shabala S, Demidchik V, Shabala L, Cuin TA, Smith SJ, Miller AJ, Davies JM, Newman IA (2006) Extracellular Ca^{2+} ameliorate NaCl induced K^{+} loss from Arabidopsis root

and leaf cells by controlling plasma membrane K^+-permeable channels. *Plant Physiol* **141**: 1653–1665

Shabala S, Mackay A (2011) Ion transport in halophytes. *Adv Bot Res* **57**: 151−199

Shabala S, Newman I (1999) Light-induced changes in hydrogen, calcium, potassium, and chloride ion fluxes and concentrations from mesophyll and epidermal tissues of bean leaves. Understanding the ionic basis of light-induced bioelectrogenesis. *Plant Physiol* **119**: 1115–1124

Shabala S, Pang J, Zhou M, Shabala L, Cuin TA, Nick P, Wegner LH (2009) Electrical signalling and cytokinins mediate effects of light and root cutting on ion uptake in intact plants. *Plant Cell Environ* **32**: 194–207

Shabala S, Pottosin I (2014) Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. *Physiol Plant* **151**: 257–279

Shabala S, Schimanski LJ, Koutoulis A (2002) Heterogeneity in bean leaf mesophyll tissue and ion flux profiles: Leaf electrophysiological characteristics correlate with the anatomical structure. *Ann Bot* **89**: 221–226

Shabala S, Shabala L (2002) Kinetics of net H^+, Ca^{2+}, K^+, Na^+, NH_4^+, and Cl^- fluxes associated with post-chilling recovery of plasma membrane transporters in *Zea mays* leaf and root tissues. *Physiol Plantarum* **114**: 47–56

Shabala S, Shabala S, Cuin TA, Pang J, Percey W, Chen Z, Conn S, Eing C, Wegner LH (2010) Xylem ionic relations and salinity tolerance in barley. *Plant J* **61**: 839-853

Shabala S, Whtie R, Djorjevic M, Ruan YL, Mathesius U (2015a) Root to shoot signaling: integration of diverse molecules, pathways and functions. *Funct Plant Biol* (http://dx.doi.org/10.1071/FP15252)

Shabala S, Wu H, Bose J (2015b) Salt stress sensing and early signalling events in plant roots: current knowledge and hypothesis. *Plant Sci* **241**: 109–119

Shah SH, Gorham J, Forster BP, Wyn Jones RG (1987) salt tolerance in the triticeae: the contribution of the D genome to cation selectivity in hexaploid wheat. *J Exp Bot* **38**: 254–269

Shakirova FM, Sakhabutdinova AR, Bezrukova MV, Fatkhutdinova RA, Fatkhutdinova DR (2003) Changes in the hormonal status of wheat seedlings induced by salicylic acid and salinity. *Plant Sci* **164**: 317–322

Shavrukov Y, Gupta NK, Miyazaki J, Baho MN, Chalmers KL, Tester M, Langridge P, Collins NC (2010) *HvNax3*–a locus controlling shoot sodium exclusion derived from wild barley (*Hodeum vulgare* ssp. *spontaneum*). *Funct Inter Genomics* **10**: 277–291

Shewry PR (2009) Wheat. *J Exp Bot* **60**: 1537–1553

Shi H, Ishitani M, Kim C, Zhu JK (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. *Proc Nat Acad Sci* **97**: 6896–6901

Shi H, Lee BY, Wu SJ, Zhu JK (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsus thaliana*. *Nat Biotechnol* **21**: 81–85

Shi H, Quintero FJ, Pardo JM, Zhu JK (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. *Plant Cell* **14**: 465−477

Shi H, Zhu JK (2002a) Regulation of expression of the vacuolar Na^+/H^+ antiporter gene *AtNHX1* by salt stress and abscisic acid. *Plant Mol Biol* **50**: 543–550

Shi H, Zhu JK (2002b) SOS4, a pyridoxal kinase gene, is required for root hair development in Arabidopsis. *Plant Physiol* **129**: 585–593

Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular response to dehydration and low temperature: differences and cross-talk between two stress signalling pathways. *Curr Opin Plant Biol* **3**: 217–223

Shimizu H, Watanabe E, Hiyama TY, Nagakura A, Fujikawa A, Okado H, Yanagawa Y, Obata K, Noda M (2007) Glial Na_X channels control lactate signalling to neurons for brain $[Na^+]$ sensing. *Neuron* **54**: 59–72

Shono M, Wada M, Hara Y, Fujii T (2001) Molecular cloning of Na^+-ATPase cDNA from a marine alga, *Heterosigma akashiwo*. *Bichem Biphy Acta* **1511**: 193–199

Silva P, Gerós H (2009) Regulation by salt of vacuolar H^+-ATPase and H^+-pyrophosphatase activities and Na^+/H^+ exchange. *Plant Signal Behav* **4**: 718–726

Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG, Roubelakis-Angelakis KA (2006) Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. *Plant Cell* **18**: 2767–2781

Slabu C, Zörb C, Steffens D, Schubert S (2009) Is salt stress of faba bean (*Vicia faba*) caused by Na^+ or Cl^- toxicity? *J Plant Nutr Soil Sc* **172**: 644–651

Smethurst CF, Rix K, Garnett T, Aurich G, Bayart A, Lane P, Wilson SJ, Shabala S (2008) Multiple traits associated with salt tolerance in lucerne: revealing the underlying cellular mechanisms. *Funct Plant Biol* **35**: 640–650

Smethurst CF, Shabala S (2003) Screening methods for waterlogging tolerance in lucerne: comparative analysis of waterlogging effects on chlorophyll fluorescene, photosynthesis, biomass and chlorophyll content. *Funct Plant Biol* **30**: 335–343

Storey R, Schachtman DP, Thomas MR (2003) Root structure and cellular chloride, sodium and potassium distribution in salinized grapevines. *Plant Cell Environ* **26**: 789–800

Suelter CH (1970) Enzymes activated by monovalent cations. *Science* 168:789–795.

Sugisawa A, Umegaki K (2002) Physiological concentrations of (–)-epigallocatechin-3-*O*-gallate (EGCg) prevent chromosomal damage induced by reactive oxygen species in WIL2-NS cells. *J Nutr* **132**: 1836–1839

Sun J, Dai S, Wang R, Chen S, Li N, Zhou X, Lu C, Shen X, Zheng Z, Hu Z, Zhang Z, Song J, Xu Y (2009) Calcium mediates root K^+/Na^+ homeostasis in poplar species differing in salt tolerance. *Tree Physiol* **29**: 1175–1186

Sunarpi, Horie T, Motoda J, Kubo M, Yang H, Yoda K, Horie R, Chan WY, Leung HY, Hattori K, Konomi M, Osumi M, Yamagami M, Schroeder JI, Uozumi N (2005) Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na unloading from xylem vessels to xylem parenchyma cells. *Plant J* **44**: 928–938

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in response of plants to abiotic stress. *Plant Cell Environ* **35**: 259–270

Sze H, Li X, Palmgren MG (1999) Energization of plant cell membranes by H^+-pumping ATPase: regulation and biosynthesis. *Plant Cell* **11**: 677–689

Takeda S, Matsuoka M (2008) Genetic approaches to crop improvement: responsing to environmental and population changes. *Nat Rev Genet* **9**: 444–457

Talbott LD, Zeiger E (1996) Central roles for potassium and sucrose in guard-cell osmoregulation. *Plant Physiol* **111**: 1051–1057

Taleisnik E, Grunberg K (1994) Ion balance in tomato cultivars differing in salt tolerance. I. Sodium and potassium accumulation and fluxes under moderate salinity. *Physiol Plant* **92**: 528–534

Tavakkoli E, Rengasamy P, McDonald GK (2010) High concentrations of Na+ and Cl- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. *J Exp Bot* **61**: 4449–4459

Tegg R, Melian L, Wilson CR, Shabala S (2005) Plant cell growth and ion flux responses to the streptomycete phytotoxin thaxtomin A: calcium and hydrogen flux patterns revealed by the non- invasive MIFE technique. *Plant Cell Physiol* **46**: 638–648

Terry N, Ulrich A (1973) Effects of potassium deficiency on the photosynthesis and respiration of leaves of sugar beet. *Plant Physiol* **51**: 783–786

Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. *Ann Bot* **91**: 503–507

Thiel G, Battey N (1998) Exocytosis in plants. *Plant Mol Biol* **34**: 111–125

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. *Proc Natl Acad Sci USA* **108**: 20260–20264

Tran LSP, Urao T, Qin F, Maruyama K, Kakimoto T, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*. *Proc Natl Acad Sci USA* **104**: 20623–20628

Trębacz K, Simonis W, Schönknecht G (1994) Cytoplasmic Ca^{2+}, K^+, Cl^-, and NO_3^- activities in the liverwort *Conocephalum conicum* L. at rest and during action potentials. *Plant Physiol* **106**: 1073–1084

Tsay YF, Ho CH, Chen HY, Lin SH (2011) Integration of nitrogen and potassium signalling. *Annu Rev Plant Biol* **62**: 207–226

Ullrich SE (2011) Significance, adaption, production, and trade of barley. In SE, Ullrich, eds, Barley production, improvement and uses, Wiley-Blackwell, Oxford, pp 3–13

Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death. *Plant Physiol* **141**: 386−390

Vandesompele J, De Paepe A, Speleman F (2002) Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real time RT-PCR. *Anal Biochem* **303**: 95–98

van Goor BJ, and van Lune P (1980) Redistribution of potassium, boron, iron, magnesium and calcium in apple trees determined by an indirect method. *Physiol Plant* **48**: 21–26

Veeranagamallaiah G, Jyothsnakumari G, Thippeswamy M, Chandra Obul Reddy P, Surabhi GK, Sriranganayakulu G, Mahesh Y, Rajasekhar B, Madhurarekha C, Sudhakar C (2008) Proteomic analysis of salt stress response in foxtail millet (*Setaria italic* L. cv. Prasad) seedlings. *Plant Sci* **175**: 631–641

Verbelen JP, De Cnodder T, Le J, Vissenberg K, Baluška F (2006) The root apex of Arabidopsis thaliana consists of four distinct zones of growth activities. Meristematic

zone, transition zone, fast elongation zone and growth terminating zone. *Plant Signal Behav* **1**: 296−304

Verdoy D, Lucas MM, Manrique E, Covarrrubias AA, De FelipeMR, Pueyo JJ (2004) Differential organ-specific response to salt stress and water deficit in nodulated bean (*Phaseolus vulgaris*). *Plant Cell Environ* **27**: 757−767

Véry AA, Sentenac H (2003) Molecular meachanisms and regulation of K^+ transport in higher plants. *Annu Rev Plant Bio* **54**:575–603

Volkov V, Boscari A, Clément M, Miller AJ, Amtmann A, Fricke W (2009) Electrophysiological characterization of pathways for K^+ uptake into growing and non-growing leaf cells of barley. *Plant Cell Environ* **32**: 1778–1790

Wada M, Satoh S, Kasamo K, Fujii T (1989) Presence of Na^+-activated ATPase in the plasma membrane of the marine raphidophycean *Heterosigma akashiwo*. *Plant Cell physiol* **30**: 923–928

Wakeel A, Farooq M, Qadir M, Schubert S (2011) Potassium substitution by sodium in plants. *Crit Rev Plant Sci* **30**: 401–413

Walker DJ, Smith SJ, Miller AJ (1995) Simultaneous measurement of intracellular pH and K^+ or NO_3^- in barely root cells using triple-barreled, ion-selective microelectrodes. *Plant Physiol* **108**: 743–751

Wang M, Zheng Q, Shen Q, Guo S (2013) The critical role of potassium in plant stress response. *Int J Mol Sci* **14**: 7370–7390

Wang R, Chen S, Deng L, Fritz E, Hüttermann A, Polle A (2007) Leaf photosynthesis, fluorescence response to salinity and the relevance to chloroplast salt compartmentation and anti-oxidative stress in two poplars. *Trees* **21**: 581–591

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* **218**: 1–14

Wang Y, Wu WH (2013) Potassium transport and signalling in higher plants. *Annu Rev Plant Biol* **64**: 451–476

Ward JM, Hirschi KD, Sze H (2003) Plants pass the salt. *Trends Plant Sci* 8 (5): 200–201.

Wegner LH, de Boer AH (1997) Properties of two outward-rectifying channels in root xylem parenchyma cells suggest a role in K^+ homeostasis and long distance signaling. *Plant Physiol* **115**: 1707–1719

Wrzaczek M, Brosché M, Kangasjärvi J (2013) ROS signalling loops – production, perception, regulation. *Curr Opin Plant Biol* **16**: 575–582

Wu H, Shabala L, Barry K, Zhou M, Shabala S (2013) Ability of leaf mesophyll to retain potassium correlates with salinity tolerance in wheat and barley. *Physiol Plant* **149**: 515–527

Wu H, Shabala L, Liu X, Azzarello E, Pandolfi, C, Zhou M, Chen, ZH, Bose J, Mancuso S, Shabala S (2015a) Linking salinity tolerance with tissue-specific Na^+ sequestration in wheat roots. *Front Plant Sci* **6**: 71

Wu H, Shabala L, Zhou M, Shabala S (2014) Durum and Bread wheat differ in their ability to retain potassium in leaf mesophyll: implications for salinity stress tolerance. *Plant Cell Physiol* **55**: 1749–1762

Wu H, Shabala L, Zhou M, Shabala S (2015b) Chloroplast-generated ROS dominates NaCl-induced K^+ efflux in wheat leaf mesophyll. *Plant Signal Behav* **10**: 5, e1013793

Wu H, Shabala L, Zhou M, Stefano G, Pandolfi, C, Mancuso S, Shabala S (2015c) Developing and validating a high-throughput assay for salinity tissue tolerance in wheat and barley. *Planta* **242**: 847–857

Wu H, Zhu M, Shabala L, Zhou M, Shabala S (2015d) K^+ retention in leaf mesophyll, an overlooked component of salinity tolerance mechanism: a case study for barley. *J Integr Plant Biol* **57**: 171–185

Wu SJ, Ding L, Zhu JK (1996) *SOS1*, a genetic locus essential for salt tolerance and potassium acquisition. *Plant Cell* **8**: 617–627

Xu R, Wang J, Li C, Johnson P, Lu C, Zhou M (2012) A single locus is responsible for salinity tolerance in a Chinese landrace barley (*Hordeum vulgare* L.). *Plos One* **7**: e43079

Xue ZY, Zhi DY, Xue GP, Zhang H, Zhao YX, Xia GM (2004) Enhanced salt tolerance of transgenic wheat (*Triticum aestivum* L.) expressing a vacuolar Na^+/H^+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+. *Plant Sci* **167**: 849–859

Yadav NS, Shukla PS, Jha A, Agarwal PK, Jha B (2012) The *SbSOS1* gene from the extreme halophyte *Salicornia brachiate* enhances Na^+ loading in xylem and confers salt tolerance in transgenic tobacco. *BMC Plant Biol* **12**: 188

Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci* **10**: 615–620

Yang Q, Chen ZZ, Zhou XF, Yin HB, Li X, Xin XF, Hong XH, Zhu JK, Gong Z (2009) Overexpression of *SOS* (*Salt Overly Sensitive*) genes increases salt tolerance in transgenic Arabidopsis. *Mol Plant* **2**: 22–31

Yang YW, Newton RJ, Miller FR (1990) Salinity tolerance in sorghum. I. Whole plant response to sodium chloride in *S. bicolor* and *S. halepense*. *Crop Sci* **30**: 775−781

Yatime L, Laursen M, Morth JP, Esmann M, Nissen P, Fedosova NU (2011) Structural insights into the high affinity binding of cardiotonic steroids to the Na^+, K^+-ATPase. *J Struct Biol* **174**: 296–306

Yeo AR, Flowers TJ (1986) Salinity resistance in rice (*Oryza sativa* L.) and a pyramiding approach to breeding varieties for saline soils. *Aust J Plant Physiol* **13**: 161–173

Yin XY, Yang AF, Zhang KW, Zhang JR (2004) Production and analysis of transgenic maize with improved salt tolerance by the introduction of *AtNHX1* gene. *J Integr Plant Biol* **46**: 854–861

Yokthongwattana C, Mahong B, Roytrakul S, Phaonaklop N, Narangajiavana J, Yokthongwattana K (2012) Proteomic anlysis of salinity-stressed *Chlamydomonas reinhardtii* revealed differential suppression and induction of a large number of important housekeeping proteins. *Planta* **235**: 649–659

Yue Y, Zhang M, Zhang J, Duan L, Li Z (2012) *SOS1* gene overexpression increased salt tolerance in transgenic tobacco by maintain a higher K^+/Na^+ ratio. *J Plant Physiol* **169**: 255–261

Zepeda-Jazo I, Velarde-Buendia AM, Enriquez-Figueroa R, Bose J, Shabala S, Muniz-Murguia J, Pottosin II (2011) Polyamines interact with hydroxyl radicals in activating Ca^{2+} and K^+ transport across the root epidermal plasma membranes. *Plant Physiol* **157**: 2167–2180

Zhang G, Miura Y, Yagasaki K (2000) Suppression of adhesion and invasion of hepatoma cells in culture by tea compounds through antioxidative activity. *Cancer Lett* **159**: 169–173

Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat Biotechnol* **19**: 765–768

Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc Natl Acad Sci USA* **98**: 12832–12836

Zhang JL, Flowers TJ, Wang SM (2010) Mechanisms of sodium uptake by roots of higher plants. *Plant Soil* **326**: 45–60

Zhao D, Oosterhuis DM, Bednarz CW (2001) Influence of potassium deficiency on photosynthesis, chlorophyll content, and chloroplast ultrastructure of cotton plants. *Photosynthetica* **39**: 103–109

Zhou G, Johnson P, Ryan PR, Delhaize E, Zhou M (2012) Quantitative trait loci for salinity tolerance in barley (*Hordeum vulgare* L.). *Mol Breeding* **29**: 427–436

Zhu JK (2000) Genetic analysis of plant salt tolerance using Arabidopsis. *Plant Physiol* **124**: 941–948

Zhu JK (2001) Plant salt tolerance. *Trends Plant Sci* **6**: 66–71

Zhu JK (2003) Regulation of ion homeostasis under salt stress. *Curr Opin Plant Biol* **6**: 441–445

Zhu M, Shabala L, Cuin TA, Zhou M, Munns R, Huang X, Shabala S (2015) Nax loci affect SOS1-like Na^+/H^+ exchanger expression and activity in wheat. *J Exp Bot* (doi: 10.1093/jxb/erv493)

Zhu M, Shabala S, Shabala L, Fan Y, Zhou MX (2016) Evaluating predictive values for various physiological indices for salinity stress tolerance in wheat. *J Agro Crop Sci* **202** 15–124

Živanović BD, Pang J, Shabala S (2005) Light-induced transient ion flux response from maize leaves and their association with leaf growth and photosynthesis. *Plant Cell Environ* **28**: 340–352

Zörb C, Noll A, Karl S, Leib K, Yan F, Schubert S (2005) Molecular characterization of Na^+/H^+ antiporters (*ZmNHX*) of maize (*Zea mays* L.) and their expression under salt stress. *J Plant Physiol* **162**: 55–66